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Immunization

Vaccine Adjuvant Delivery System and Strategies

Edited by Ning Wang and Ting Wang

IMMUNIZATION - VACCINE ADJUVANT DELIVERY SYSTEM AND STRATEGIES

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Meet the editors

Ning Wang, an Associate Professor in Hefei University of Technology in China, obtained her PhD degree in 2014, majored in pharmaceutics, from Shenyang Pharmaceutical University. Dr.Wang focuses her research interests on the targeted delivery of subunit vaccines and chemical drugs using lipidic nanocarriers and excels in preparing liposomes as a drug/vaccine carrier and constructing nanoparticle-based vaccine adjuvant-delivery systems (VADS). Dr. Wang has gained several competitive research grants on VADS funded by the National Natural Science Foundation of China and Provincial Natural Science Foundation. She has published more than 20 full research and review papers and 3 chapters of 2 books, and has been granted 2 patents.

Ting Wang, a Professor and Director of Pharmaceutics at the Anhui Medical University, obtained his PhD degree in pharmaceutics in 2007 from the Shenyang Pharmaceutical University of China. His research interests involve constructing vaccine adjuvant-delivery systems (VADS) and targeted drug delivery systems with self-assembly nanocarriers, including liposomes, phospholipid bilayer-coated alum nanoparticles, anhydrous reverse micelles and lipid-inorganics hybrid nanoparticles, to resolve challenging problems confronted in the fields of vaccine/drug development, including targeted delivery, insoluble drug oral absorption and mucosal vaccination. He has won several competitive research grants funded by the National Natural Science Foundation of China and Provincial Natural Science Foundation, several national and local prizes, and has been granted 5 patents, and published more than 50 full research/review papers and 6 chapters of 5 books.

Contents

Preface

Nowadays, immunization still plays a crucial role in maintaining human health, as it can be effectively employed for prophylaxis of various dangerous infections and erase many life-threatening diseases. Thus, knowledge on immunization appears important not only to professionals who can use it for better carrying out their special work, but also to the general public, who will be readier to accept immunization after making acquaintance with the relevant information. In this book, authors from different countries introduced state-of-the-art advances in VADSs that are developed with various NPs, such as liposomes, non-degradable inorganic NPs (iNPs), VLPs, emulsions, ISCOMs and polymeric NPs, for treatment of different types of diseases, mainly infections and cancer. The content includes: design principles, formulations and rationales of these NPs, which have been devised as an effective VADS able to stimulate potent Ag-specific humoral and cellular immune responses; the applications of VADSs in infection prophylaxis, as well as cancer immunotherapy; problems and their resolution in both human and poultry immunization; and, also, the mathematical model for assay of the basic immunization problem (BIP) that is observed from a financial point of view.

Wu et al described the VADS constructed with the small molecule-self-assembled NPs, such as inorganic NPs (iNPs), emulsions, liposomes, and ISCOMs, which are well designed for the development of subunit vaccines able to not only deliver vaccine ingredients to immune cells but also direct the immunoresponse toward a Th1 and Th2 balanced pathway to establish both humoral and cellular immunity.

Liu and coworkers introduced NPs formulated with polymeric materials, such as poly(lactic-co-glycolic acid), viral proteins, chitosan, hyaluronic acid, and polystyrene, with some also bearing intrinsic adjuvanticity, which are widely employed as a VADS and have shown great potential in developing various subunit vaccines. Particularly, the polymeric NPs engineered with functional materials possess many features, such as targeting delivery, lysosome escape, anti-damaging protection and ability to guide immune reactions toward a Th1 and Th2 pathway, which are crucial for establishing humoral and cellular immunity.

Shen and colleagues presented a chapter on VADS used for cancer immunotherapy, which has recently been rapidly developed, at least partially, as a result of the advancement in both exploring powerful tools for identification of tumor-associated antigens (tAgs) and revealing the mechanisms underlying immunoresponses toward tumors. Notably, cancer immunotherapy has already shown remarkable therapeutic potentials, as evidenced by the striking outcomes of clinical applications of the adoptive cell transfer (ACT) and the immune checkpoint inhibitors, which has significantly encouraged researchers to formulate tAgs with NPs to form a VADS and to provide an alternative way to enhancing the efficacy

of cancer immunotherapy as well as to mitigate the off-target toxicity of tAgs. Especially when engineered with some special functions, such as targeting APCs, reeducating tumor-associated macrophages with tumor-suppressing properties, and triggering CTLs in large numbers, the NC-VADSs show a big potential in killing tumor cells.

Shaikh provided a chapter aiming at bridging the information gaps about system-level factors that are currently impeding the optimal delivery and uptake of immunization services to children through the Expanded Program on Immunization (EPI). In this chapter, the author drew the thematic content based on not only a critical review of the EPI-related international and national reports but also consulting government reports, surveys and publications on health system to generate from the literature the views on financing, governance, service delivery, human resources, information system, and supplies and vaccines, to make certain crucial conclusions that are useful for setting up EPI with a better immunization coverage, EPI operations and performance and can help in developing a more reasonable EPI.

Sharif emphasized the great importance of poultry immunization, which is of significance for the poultry sector since it provides an economical and efficient way for protection of poultry from lethal infectious diseases. The author reported that, in Pakistan, the poultry sector is of big significance in terms of production of food items such as meat and eggs and is, however, as the second most important sector after the textile industry, also encountered with the challenge of the high incidence of disease outbreaks, which will inevitably result in colossal economic losses. The diseases of commercial and rural poultry include mainly Newcastle disease (ND), infectious bursal disease (IBD), fowl pox, Marek's disease, infectious bronchitis (IB), avian influenza, and hydropericardium syndrome, of which the outbreaks have also occurred in vaccinated flocks. The author comprehensively reviewed the causes of the immunization failure, which are useful for identifying the prophylactic measures regarding disease outbreaks in poultry flocks, and further highlighted the procedures for successful immunization.

In the last chapter, Zaremba presented a mathematical model for assay of the basic immunization problem (BIP) that is understood from a finance point of view with key notions, such as duration and convexity, and illustrated with recently obtained results for guaranteeing immunization. The introduction of a mathematical model may trigger big interest among medical scientists to handle BIP with an alternative strategy, though a big challenge may confront medical researchers in understanding and applying the mysterious mathematical formulas for handling BIPs, e.g., the author explained, with numerous mathematical equations, that BIP relies on a construction of such a bond portfolio (BP), meaning a selection of individual bonds with the present value of C dollars that the single liability to pay L dollars q years from now, will be discharged by means of BP (a patient, due to paid immunization, will return to health at time q), no matter what random shift a(t) of current interest rates s(t) (a particular disease) will occur in the future. Nevertheless, the author suggested that certain finance notions, such as duration and convexity of a bond portfolio, might give extra insight to medical researchers working in the immunization area both into BIP from a finance viewpoint and into similar problems in medicine. Also, considerable attention is also paid in this chapter to certain mathematical notions (linear independence of vectors, base of a linear space, a Hilbert space, triangular functions) because of their successful applications to solving problems in bond portfolio for immunization.

In summary, this book written by authors from several countries introduces comprehensive knowledge on immunization, including state-of-the-art advances in VADSs; the applications of VADSs for prophylaxis of infectious diseases as well as cancer immunotherapy; the problems and their resolutions in both human and poultry immunization; and also, the mathematical model that can be used for assay of the basic immunization problem (BIP) understood from a finance point of view. Therefore, this book will present a useful reference on immunization, VADSs and related strategies for a wide range of readers.

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Chapter 1

Introductory Chapter: Immunization - Vaccine Adjuvant Delivery System and Strategies

Ning Wang and Ting Wang

Additional information is available at the end of the chapter

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1. Introduction

Immunization plays a key role in maintaining human health as it saves millions of lives in the most economical way from lethal pathogens and other fatal diseases each year, thanks to the advanced development of model vaccines, which are biological preparations containing an antigenic agent that resembles a disease-causing microorganism to stimulate the host's immune system, thus providing active acquired immunity to a particular disease and destroying it [1, 2]. Since Jenner's pioneering inoculations in the late eighteenth century, vaccines have been successfully developed to combat various diseases and each year saved numerous lives from, mostly, lethal infections and now also certain cancers [3, 4]. Especially, taking advantage of the tools discovered in microbiology and immunology, vaccines have recently obtained great achievements as demonstrated by their successful performances in conquering some formidable pathogens, such as smallpox and rabies, which are used to claim many lives. However, the list of pathogens for which there exist no vaccines is still long, and, in particular, many pathogens, such as human immunodeficiency virus (HIV), herpes simplex virus (HSV), and Ebola virus (EBV), are still posing a big threat to human life, therefore needing urgently the effective products to cope with their infections [5].

Vaccines can stimulate the host immune system to develop an armament of immunity capable of clearing the abnormalities after administration, because they are developed with the antigenic components that are featured by pathogens or neoplasms and usually include three types: the live attenuated microbes, killed microbes, and just purified antigens (Ags) of microbes or neoplasms [6]. The former two consisting of live attenuated or killed microbes are the conventional vaccines with high immunogenicity but, unfortunately, are also linked to a relatively poor safety profile as they possess the potential to revert the virulence and induce the drifted immune responses leading to incontrollable immunity as well as unacceptable inflammations. In contrast, the third one with purified Ags, called a subunit vaccine, has

defined components to induce immune responses aiming just at the matched targets causing few safety concerns and thus can be employed to fight the infectious pathogens as well as malignant neoplasms that are carrying the identical Ags [7]. Presently, subunit vaccines are attracting more and more research interests owing to also their diverse applications, adaptive functions, and numerous advantages over the whole microbe-based conventional ones, and these aspects may well be comprehensively summarized as follows [8]:

1. Their defined noninfectious components effectively confine capricious reversion to virulence while reducing significantly the risk of allergic or autoimmune response [9].
2. Their production may avoid the use of dangerous microorganisms but may be carried out with solid-phase peptide synthesis in a reproducible, scalable, and economical manner, providing an alternative tool to obtain products to conquer certain pathogens that are problematic to culture (e.g., sporozoites for malaria vaccines) for attenuation of virulence [10].
3. Ags in subunit vaccines are generally water-soluble allowing them to form a solution together with cryoprotectants such as disaccharide, followed by freeze-drying into the stable anhydrous fitting storage and transportation in a controlled temperature chain (CTC) or completely at room temperature [11].
4. Also, subunit vaccines can be tailored with certain pathogen-/damage-associated molecular patterns (PAMP/DAMP) to be recognized by APCs for efficient activation, even including several peptide epitopes targeting different stages in the life cycle of a pathogen [8, 12, 13]; obviously, this is particularly useful for developing anticancer vaccines, wherein whole protein can hardly be used due to its similarity to the endogenous human proteins and carcinogenic properties [14].

However, unfortunately, subunit vaccines often have a rather weak immunogenicity, due to lack of the immunostimulatory components broadly shared by pathogens while being distinguishable from host molecules, which are collectively referred to as pathogen-associated molecular patterns (PAMPs) [15] able to bind to and trigger mammalian pattern recognition receptors (PRRs), such as the TLRs (toll-like receptors), NOD-like receptors (the nucleotide-binding oligomerization domain-like receptors), RIG-I-like receptors (retinoic acid-inducible gene-I-like receptor), and C-type lectin receptors, thus playing an adjuvant role to activate the innate immunity, followed by sponsoring a series of adaptive reactions involved in establishing the Ag-specific immunity [16].

Thus, while subunit vaccines are regarded as a safer product than the whole microbe-based conventional ones, they are also poorly immunogenic and often require an adjuvant or a vaccine adjuvant-delivery system (VADS) able to target the professional Ag-presenting cells (APCs), such as dendritic cells (DCs) and macrophages (MPs), to make full use of Ags and boost their immunostimulatory activity [17–21]. A vaccine adjuvant is defined as a non-specific immunopotentiating substance but capable of enhancing the body's immune response to the Ag or changing the type of immune responses, when administered either alone in advance or simultaneously together with the vaccine Ag. Although its immune-boosting mechanisms remain elusive, an adjuvant is argued to fulfill the functions involving, roughly, two aspects: (1) generating damages on host cells/tissues, thus sending dangerous signals out to activate

the immune system and (2) binding to PRRs and exciting the innate immune cells, such as DCs, MPs, histiocytes, and mast cells, which subsequently initiate the innate immune responses to sponsor the subsequent adaptive immune responses [22, 23]. Accordingly, vaccine adjuvants may well be classified into two types: type I, the natural or synthetic substances with intrinsic adjuvanticity, squalene/squalane, saponin, chitosan, hyaluronic acid (HA), and various pattern recognition receptor agonists (PRRas) and type II, the micron-/nanometer-sized particles, such as alum (insoluble aluminum salt) and vaccine adjuvant-delivery systems (VADSs) that are carriers engineered with at least two fundamental functions, i.e., adjuvanticity and Ag delivery. VADSs are usually constructed with a variety of biocompatible nanoparticles (NPs) made of various organic or inorganic materials, such as liposomes, ISCOMs (immune-stimulating complexes), polymeric NPs, VLPs (virus-like particles), emulsions, and the inorganic NPs, which are often incorporated with type I substances to further enhance their immunopotentiating functions [6, 24].

2. Immune responses for establishing the Ag-specific immunity

Always confronting and fighting with dangerous pathogens, mammals have gradually evolved to form a complexed defensive immune system, which can be classified into subsystems of the innate immune system versus the adaptive immune system [25]. The innate immune system consists of surface barriers, complement system, and various leukocytes including the phagocytes (macrophages, neutrophils, and dendritic cells), innate lymphoid cells, mast cells, eosinophils, basophils, and natural killer cells, which fulfill the role of non-specific immune defenses responding to pathogens in a generic way conferring short-lasting immunity against a pathogen [26]. For this, mammalian leukocytes are evolutionarily equipped with receptors able to recognize certain pathogen components bearing specific structural characteristics, such as free bacterial and viral DNA, lipoproteins, lipopolysaccharides, and flagellins, which are pathogen-/danger-associated molecular patterns (PAMPs/DAMPs) [27]. These functional receptors are expressed by host immune cells, such as TLR1 to TLR13, NOD-like receptors, RIG-I-like receptors, and C-type lectin of mannose receptors, which are collectively called pattern recognition receptors (PRRs), with each capable of selectively binding to specific PAMPs/DAMPs of pathogens, leading to the activation of the innate immune cells, which subsequently sponsor the immuno-responses of the whole immune system, thus providing the bases for defending against pathogens [28].

However, establishing the Ag-specific immunity for defending against pathogens involves several complex immune pathways going with the orchestration of numerous immunocytes, cytokines, and chemokines and starts, usually, upon the activation of APCs for innate immune reactions triggered by their internalized antigenic substances (Ags) that they distinguished as dangerous signals through, in most cases, the process of PRR-PAMP/DAMP recognition [23]. Briefly, positioned at the frontier of pathogen/vaccine recognition, APCs first take up, in a size-dependent manner (e.g., NPs with a size of <150 nm are taken up by APCs by clathrin-mediated endocytosis, while microparticles by phagocytosis); the Ags appeared in peripheral tissues or in the draining lymph nodes (dLNs), wherein APCs will mature and process the

internalized Ags into pieces with epitopes, which are finally bound to MHC-II (major histocompatibility complex II) and/or MHC-I and displayed on cell surfaces for presentation to T cells [29]. MHC-I molecules are assembled in the endoplasmic reticulum (ER) with stabilization by chaperone proteins (including calreticulin, Erp57, protein disulfide isomerase (PDI), and tapasin) and are loaded, under tapasin mediation, with exogenous (viral or self-originated) Ags, which are translocated from the cytoplasm into the ER by TAP (transporter associated with antigen presentation) for presentation via T-cell receptors (TCRs) to $CD8^+$ T cells for their activation [30]. MHC-II molecules are assembled in the ER, stabilized by an invariant chain (Ii) and transported through the Golgi to fuse with a late endosome forming the MHC-II endosome compartment (MIIC) with an acidic interior containing proteases cathepsin S and cathepsin L, which when activated will digest Ii, leaving in the peptide-binding groove of the MHC-II a residual class II-associated Ii peptide (CLiP), which later is exchanged for an antigenic peptide (usually exogenous Ags) derived from a protein degraded in the endosomal pathway for presentation via T-cell receptors (TCRs) to $CD4^+$ T cells for their activation [30].

During Ag presentation, a substantial number of various signaling cytokines such as interleukins and interferons, as well as chemokines, are secreted by matured APCs and other immunocytes to promote the MHC-II-Ag epitope-triggered $CD4^+$ T-cell differentiation into either T-helper type-1 (Th1) cells that will secrete IFN-γ, IL-12, and IL-2 or Th2 cells that will secrete IL-4. Then, Th1 cells will further secrete IL-12 and IFN-γ facilitating the MHC-I-Ag epitope-triggered $CD8^+$ T cells to proliferate and differentiate into the Ag-specific cytotoxic T lymphocytes (CTLs), thus establishing the cellular immunity while forming memory T cells [31, 32]. Meanwhile, the Th2 cells mature to favor Ag presentation to B cells via B cell receptors (BCRs) and to secrete IL-4, IL-6, and IL-10, which are also beneficial for promoting the Ag-activated B cells to proliferate and differentiate into plasma cells to produce the anti-Ag antibodies, establishing finally the humoral immunity while forming memory B cells [33]. With memory T and B cells, upon encountering pathogens, both humoral and cellular immunity can be rapidly established; while humoral immunity neutralizes the extracellular pathogens during or before the infection and thus is fitting for the prophylaxis of pathogen invasion, cellular immunity is mainly responsible for destroying already infected or abnormal human cells and therefore may be employed for the clearance of the cell-hidden pathogens or malignant tumors, through specialized TCR recognition of the precisely matched Ags nestled in the groove of MHC-I on cells [12].

3. Immunization: vaccine adjuvant-delivery system and strategies

In designing the NP-based VADSs, what should be emphasized is that the differently sourced Ags are processed and trigger immune signal transduction in different ways [34]. Usually, the internalized exogenous Ags delivered by NPs are processed intracellularly by APCs into small antigens just inside the endolysosomal vesicles and then loaded favorably on MHC-II molecules, leading to activation of CD4+ T cells to differentiate into Th2 cells, which will further stimulate production of antibodies by B cells [8]. In contrast, the endogenous Ags, such as viral Ags, cancer components, and intracellular-degraded proteins, are usually presented in the cytosol and often

loaded on MHC-I molecules, allowing for further activation of $CD8^+$ T cells to differentiate into CTLs and to engender cellular immunity [35]. However, it is generally believed that, though not well understood, provided the exogenous Ags are transported via membrane fusion or other ways engendering Ag lysosome escape into the cytosol, they can also be processed via MHC-I presentation in just the same manner as that for endogenous Ags, and this process is known as Ag cross presentation [36]. This provides the basis for designing the NP-based VADS which are exogenous particles favoring of inducing humoral immunity but may be adorned with materials that can facilitate APC internalization in the membrane fusion manner or promote endolysosome escape and cross presentation of Ags to induce cellular immunity, thus expanding VADS into various applications, including mainly prophylaxis of infections, treatment of autoimmunity diseases, and immunotherapy of cancer [12]. In particular, as a multifunctional VADS, NPs modified with different materials with intrinsic and specific adjuvanticity, such as TLRas (e.g., MPL and CpG ODN), squalene, and saponin (e.g., water-soluble QS-21), though showing individually distinctive features, share some key characteristics in immune-boosting functions [37]. Summarily, these multifunctional Nps as a VADS trigger immunoresponses with features including, mainly, early activation, though at different levels, of innate immunity, which will subsequently translate into strong antibody and cellular responses to the delivered antigens [5]; a wide breadth of adaptive immunity is able to confer protections against heterovariants of pathogens; for example, vaccines delivered by liposomes containing MPL can defend against influenza viruses or human papillomavirus (HPV) strains that are not contained in the vaccines [38]; significant enhancement of the immunoresponses and the efficacy of vaccines in the elderly who show a waning immune responsiveness to infection and vaccination, as shown for vaccines formulated with MPL-liposomes against herpes zoster virus [39]. These results, together with the feasibility of large-scale manufacture and the track records of acceptable safety profiles of many liposome-based medications, pave the way for developing novel multifunctional liposomes to be used as a VADS for producing the vaccine products fitting humans of different age against infections with a high toll of morbidity and mortality.

Conflict of interest

All the authors declared no conflict of interest.

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Chapter 2

Vaccine Adjuvant Delivery Systems Constructed Using Biocompatible Nanoparticles Formed through Self-Assembly of Small Molecules

Ting Liu, Rui Qian, Qingchuan Liu, Tingni Wu and Jialong Chen

Additional information is available at the end of the chapter

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Abstract

Subunit vaccines are playing a critical role in controlling numerous diseases and attracting more and more research interests due to their numerous advantages over conventional whole microbe-based vaccines. However, subunit vaccines are weak immunogens and thus have limited capacity in eliciting the humoral and cellular immunity against pathogens. Recently, nanoparticles (NPs) formed with certain small molecules through self-assembly have been employed as an effective carrier for subunit vaccines to play roles of adjuvant, delivery and stabilization of antigens, thus engendering a vaccine adjuvant-delivery system (VADS), which shows promises to overcome the hurdles in developing subunit vaccines. In particular, the small molecule-self-assembled NPs as a VADS can not only deliver vaccine ingredients to immune cells but also influence the immunoresponse toward a Th1 (type 1 T helper cell) and Th2 balanced pathway to establish both humoral and cellular immunity. This chapter describes the innovative VADSs based on the small molecule-self-assembled NPs, such as metal NPs (mNPs), emulsions, liposomes, and ISCOMs, which are elaborately designed for the development of subunit vaccines.

Keywords: nanoparticle, self-assembly, immune response, mucosal vaccination, cellular immunity, nanocarrier, inorganic particle, danger-associated molecular pattern, targeted delivery

1. Introduction

Modern vaccine development began from the use of vaccinia against smallpox by British physician Edward Jenner in the late eighteenth century and ever since has brought many products that have saved countless human lives from being claimed by numerous infectious pathogens, such as smallpox, measles, and rabies [1]. However, many pathogens such as human immunodeficiency virus (HIV), herpes simplex virus (HSV), and Ebola virus (EBV), still lack vaccines and are still posing a great threat to human health and life [2].

Vaccines are developed based on the antigenic components, which can stimulate the host immune system to set up immunity able to clear the abnormalities and usually include three types: the live attenuated whole microbe vaccines, the killed whole microbe vaccines, and the antigenic component-based subunit vaccines [3]. The former two types are regarded as the conventional vaccines with a high capacity of defending against deleterious organisms but, unfortunately, are also linked to a relatively poor safety profile due to their possible reversion of virulence and induction of deviated immunoresponses leading to unprotective and even harmful immunity and unacceptable inflammation. In contrast, the subunit vaccine is elaborately formulated with defined components including antigen (Ags) to induce immunoresponses which is accurately targeting the matched objects, thus causing few safety concerns and can be employed to fight both infectious pathogens and detrimental neoplasms carrying the identical Ags. Subunit vaccines since the notion emergence have attracted great research interests with numerous visible and all imaginable advantages [4], including high safety without reversion to virulent state; their production needing no dangerous microorganisms; providing an alternative solution to the problematic culture for attenuating some pathogens; low risk of allergic or autoimmune reactions; customization to recognize certain pathogen-associated targets; feasible developing anticancer vaccine; carrying several peptide epitopes targeting different stages in the life cycle or subtypes of a pathogen; production in large scale in a pure state, in an economically and highly reproducible manner; high solubility allowing lyophilization to form stable dry products [4].

However, subunit vaccines often show a weak immune induction potency, due to lack of a large fraction of components associated with pathogen structural characteristics, which are broadly shared by pathogens but distinguishable from host molecules, collectively referred to as pathogen-associated molecular patterns (PAMPs) and able to activate the pattern recognition receptors (PRRs), such as the toll-like receptors (TLRs), the nucleotide-binding oligomerization domain-like receptors (NOD-like receptors), retinoic-acid-inducible gene-I-like receptors (RIG-I-like receptors), and C-type lectin receptors, to trigger as immunostimulators or an adjuvant mammalian innate immunoresponse sponsoring a series of adaptive reactions required for establishing the Ag-specific immunity [5]. Thus, while subunit vaccines are usually safer and less reactogenic than the whole organism vaccines, they often need an adjuvant or a vaccine adjuvant delivery system (VADS) to synergistically stimulate professional Ag-presenting cells (APCs) such as dendritic cells (DCs) and macrophages (MPs) for enhancing their immunization efficacy [6–10]. An adjuvant is a non-specific immune-potentiating substance, which is capable of enhancing the body's immune

response to the Ag or changes the type of immune response with some mechanisms that are not exactly known but are argued relevant to two fundamental aspects: (1) giving off dangerous signals by imposing damages on cells/tissues to activate the innate immune cells; (2) exciting PRRs of the innate immune cells, such as DCs, MPs, histiocytes and mast cells, to sponsor the subsequent adaptive responses [11, 12]. The available adjuvants are mainly micron- or nanometer-sized particles or aggregates which may be classified into two types: (1) the natural or synthetic substances with intrinsic adjuvanticity, such as alum (insoluble aluminum salt), squalene/squalane, saponin, chitosan, hyaluronic acid (HA), and various pattern recognition receptor agonists (PRRas); (2) functional carriers capable of playing roles of both adjuvant and delivery, thus regarded as vaccine adjuvant delivery system (VADSs), which are often formulated with nanoparticles (NPs) fabricated with various biocompatible materials, such as liposomes made of phospholipids and cholesterol, immune stimulating complexes (ISCOMs) of saponin and lipids, polymeric NPs made of PLGA or polystyrene, virus-like particles (VLPs) made of viral proteins, emulsions made of squalene and surfactants, and the metal NPs (mNPs) made of aluminum or gold metal compounds, which prove an efficient VADSs able to enormously enhance vaccination efficacy [3, 13].

In this chapter, we describe design principles, main formulations and the state-of-the-art advances in developing novel VADSs constructed with different types of NPs, which are formed through small molecule self-assembly and herein, include liposomes, nondegradable inorganic metal NPs (mNPs), emulsions, ISCOMs. These small molecule-based NPs have been devised as a VADS with the potential to stimulate the Ag-specific humoral and cellular immune responses and are promising in preparation of next generation vaccines against a range of infectious pathogens.

2. VADS constructed with different types of NPs formed by self-assembly of small molecules

2.1. VADS constructed with metal nanoparticles (mNPs) formed by self-aggregation

Alum is the micron-sized aggregates of water-insoluble aluminum salts and is the first substance that was discovered able to boost the efficacy of vaccines and coined the term "vaccine adjuvant," a concept which was put forward when scientists came to realize that certain materials irrelevant to pathogens but able to enhance immunoresponse induced by vaccine [14]. Since 1926, when first being used as an adjuvant, alum, such as aluminum phosphate, aluminum potassium sulfate, and aluminum hydroxide had been the only clinically used adjuvant in many subunit vaccines as well as the inactivated pathogen-based vaccines, until the approval of adjuvant calcium phosphate in diphtheria/pertussis (DT) vaccines [15]. Subsequently, an O/W nanoemulsions formed of squalene/Span 85/Tween 80, called MF59® was marketed as a VADS for delivering an influenza vaccine (Fluad®) in 1997, followed by AS04 (MPL/alum mixture) for delivering human papillomavirus (HPV, Cervarix®) and hepatitis B virus (HBV, Fendrix®) vaccines [16]. Alum forms a micron-sized VADS by just mixing the insoluble salt with other vaccine components or Ags and tends to function eliciting humoral over cellular immunity when

intramuscularly administered to humans. Though having successfully been used for nearly a century in human vaccines against numerous infectious diseases, such as hepatitis A and B, diphtheria-tetanus-pertussis (DTaP, Tdap), Haemophilus influenzae type b (Hib), HPV, and pneumococcus infection, alum is still argued to be associated with a potential risk for causing autoimmunity, long-term brain inflammation and neurological complications, as evidenced by the observation of severe disorders in recipients of alum-adjuvanted vaccines [17].

Thus, frustrated by the reactogenicity and the injury adverse effects associated alum while expected to enhance its capability to induce humoral and even cellular immunoresponses, researchers have for years endeavored to reshape the micron-sized salt adjuvant in mainly two ways: forging the micron-sized salt into NPs and coating surfaces with biocompatible materials. Recently, to develop an effective HIV vaccine, which is known a huge challenge almost since this virus discovery, Neutra's group conjugated peptide epitopes derived from HIV-1 gp120 glycoprotein to the Al_2O_3 NPs with a size of about 350 nm, which showed able to stimulate the moderate antibody responses after intraperitoneal injection but failed to stimulate mucosal immunity [18]. Also, Cui's group engineered 112 nm-sized aluminum hydroxide NPs and aluminum oxyhydroxide nanosticks with a length of 80 nm, long aspect ratio of 10 and low degree of crystallinity and showed that both aluminum NPs were able to facilitate in vitro APC uptake of the loaded protein Ags and induced in mice a stronger Ag-specific antibody response but milder local inflammation in the injection sites, compared with traditional aluminum microparticles [19, 20]. Furthermore, aluminum NPs proved able to stimulate in vitro APCs to produce uric acid, and, when injected into peritoneal cavity of mice, induced production of increased levels of uric acid, to contrast micron alum which did not in either case. The results suggest that aluminum has a stronger adjuvant activity in the form of NPs, as opposed to microparticles, may be partially attributed to their higher ability to induce endogenous danger signals such as uric acid [21].

Based on the phosphophilicity of aluminum, Wang and coworkers engineered the phospholipid bilayer-coated aluminum nanoparticles (PLANs) formed via chemisorption between phospholipid and aluminum using a procedure of reverse ethanol injection-lyophilization (REIL) [7]. The researchers demonstrated that the anhydrous Ag-PLANs had a high stability satisfying the prerequisite requirements for distribution with the controlled temperature chain instead of the integrated cold chain [22] and that upon rehydration the Ag-carried PLANs could be instantly reconstituted to form an aqueous dispersion maintaining vaccine activity. Further exploration confirmed that the PLANs remarkably enhanced APC uptake of the delivered vaccines and when given subcutaneously to mice, induced more robust Ag-specific humoral as well as cellular immunoresponses, while stimulated less local inflammations, in comparison to microparticle alum, proving that the PLANs are an efficient VADS and possess numerous advantages over alum, which has been the widely used for clinical immunization for nearly a century [7].

In recent years, other types of NPs made of metal substances, such as calcium and gold, have also become a popular VADS, owing to their certain unique physicochemical properties including inertness with good biocompatibility, facile surface modification with functional molecules, and easy size and shape control. Chiu and coworkers coated the 25 nm-sized amorphous cores of calcium phosphate nanoparticles (CaP-NPs) with peptide Ags, thus

producing a particulate vaccine with a hydrodynamic size of 60 nm and found that the small core-shell assemblies induced in mice a 3-fold increase of anti-Ag titers 3 weeks post-injection, compared to a commercial aluminum phosphate adjuvant, suggesting that CaP-NPs may be an effective VADS delivery of vaccines [23]. Morcol et al. demonstrated that CaP-NPs were also a good VADS for the inactivated influenza A/CA/04/2009 (H1N1pdm) vaccine and could enormously boost production in the intramuscularly vaccinated mice of hemagglutination inhibition (HAI), virus neutralization (VN), and IgG antibody titers, at all dose levels, relative to the nonadjuvanted vaccine. In particular, the CaP-NP vaccine equally protected mice against influenza virus at 1/3 of the Ag dose of the nonadjuvanted or alum-adjuvanted vaccines, indicating that CaP-NPs are an promising VADS which may play a crucial role in production of a dose-sparing vaccine which is of a great importance during, in particularly, an influenza pandemic [24]. Also, Powell and coworkers constructed calcium carbonate NPs which had an average diameter of 200 nm and based on opposite charge attraction, coated with polylysine and polyglutamic acid and showed that this type of the $CaCO_3$ NP-based VADS could efficiently facilitate maturation of DCs, which were simultaneously induced capable of cross-presentation of Ags. Notably, after a single injection in mice, $CaCO_3$ NPs induced strong humoral and cellular immunity without triggering secretion of inflammatory cytokines, proving $CaCO_3$ NPs are an efficient and safe VADS [25].

Gold nanoparticles (AuNPs) have unique physicochemical properties, such as an ultra-small size, large surface area to mass ratio, and high surface reactivity, presence of surface plasmon resonance (SPR) bands, biocompatibility and ease of surface functionalization, allowing this type of mNPs able to act as a versatile VADS bearing numerous beneficial features including, particularly, targeted delivery and stimulus-sensitive release. Chen et al. engineered gold NPs (AuNPs) with sizes ranging from 2 to 50 nm conjugated with foot-and-mouth disease virus associated peptide Ags and proved that gold NPs with a size of ranging in 2–17 nm induced strong humoral response, which was correlated to spleen uptake of gold NPs [26]. Gill's group prepared gold NPs conjugated with M2e peptide, an extracellular domain of influenza A virus ion channel membrane matrix protein 2 (M2e) and demonstrated that intranasal administration to mice of AuNP-M2e plus soluble CpG induced lung B cell activation and robust serum anti-M2e antibody response, resulting in high levels of both IgG1 and IgG2a subtypes [27]. Also, the group revealed that the antibodies generated by AuNP-M2e/CpG stimulation could bind to the homotetrameric form of M2 expressed on Madin-Darby canine kidney (MDCK) cells, which as an immunosorbent had been infected with H1N1, H3N2 or H5N1 strain of influenza viruses. Moreover, mice intranasally immunized with AuNP-M2e/CpG obtained 100, 92, and 100% protection against lethal challenges with A/California/04/2009 (H1N1pdm) pandemic strain, A/Victoria/3/75 (H3N2), and the highly pathogenic avian influenza virus A/Vietnam/1203/2004 (H5N1), respectively, proving AuNP-M2e/CpG a promising VADS for developing a universal influenza vaccine, a desired Holy Grail for controlling the most prevalent infections [27].

2.2. VADS constructed with emulsions formed by self-assembly of surfactants

Emulsions are formed of two immiscible liquid phases, generally oil phase and water phase, with one phase organized into small droplets (inner phase), which, depending on composition and manufacturing process, have a size in a range of from tens of nanometers to several

microns, and are dispersed in a distinct continuous phase (outer phase) under stabilization by an interfacial surfactant layer. Emulsions, based on structural characteristics, are made of three classical types of single emulsions, double emulsions and Pickering emulsions: single emulsions include oil-in-water (O/W) type denoting oil droplets being emulsified in a bulk aqueous phase, and vice versa, the water-in-oil (W/O) type; double emulsions include O/W/O and W/O/W emulsions; and Pickering emulsions are a special type with an emulsifier of solid NPs replacing surfactants [28].

Notably, the emulsions formed of special oils, such as lanolin oil, cottonseed oil, and paraffin oil, were found, like alum, in some serendipitous way, of adjuvanticity in the early twentieth century and have ever since been widely used as a VADS to produce vaccines against pathogens. For example, lipovaccines used in the 1920s were in fact the formulations consisting of killed bacterial vaccines suspended in lanolin or cottonseed oils and proved able to induce immunoresponses with additional functions of dose spare and stability enhancement [29]. Freund adjuvants are the mostly known potent emulsion-based VADS including two types: incomplete Freund adjuvant(IFA), which is essentially a viscous crude W/O emulsion containing Ags in water phase using mineral paraffin as an oil phase and mannide monooleate as a surfactant; complete Freund adjuvant (CFA), which forms by addition to IFA of heat-killed mycobacteria (*Mycobacterium tuberculosis*) and has thus a high immunostimulating potency but also a high reactogenic toxicity, rendering the adjuvant to be used only in veterinary vaccines [30]. Though IFA is rather safe compared to CFA and was actually administered to hundreds of thousands of humans as an adjuvant in polio and influenza vaccines in the mid-twentieth century, the severe local reactogenicity excluded the adjuvant from continuing clinical use [31].

Discarding the flaws of unacceptable toxicity and uncertain component associated with early emulsion adjuvants, modern emulsions as a VADS are usually formulated with well-defined factors, such as particle size, component and concentrations, and compatibility with antigens as well as human bodies, which are related to efficacy, safety, and stability [32]. Important lessons highlighted by early emulsion vaccines and deep insights into problems arising in use of the adjuvant inspired researchers to commit to developing an emulsion VADS with clear thoughts in several issues: (1) using biodegradable oil and the surfactants with an established safety profile in humans; (2) using the O/W instead of W/O emulsions to lower oil content for enhancing tolerability as well as the ease of use due to reduced viscosity; (3) enhancing potency with emulsions having a size <500 nm to promote APC uptake. As a result of the efforts directed toward these aspects, a breakthrough was made in the development of emulsion VADS in the 1980s when the squalene was explored as the oil phase of emulsions, which were thus rendered with an acceptable reactogenicity profile and potent adjuvant effects and were subsequently licensed as several proprietary products, including MF59 by Novartis, AS03® by GSK and AF03 by Sanofi Pasteur. MF59 is an O/W emulsion which is produced with a microfluidizer (MF) and contains squalene oil droplets stabilized by surfactants Tween 80 and Span85 guaranteeing the size of 160 nm for sterilization by filtration and as a VADS has proven of potent immunogenicity and low reactogenicity for a range of Ags [16]. MF59 can induce robust immunoresponses through triggering vaccinated tissue-resident immune cells to secrete a number of chemokines, which recruit other immunocytes to amplify the chemokine gradient, resulting in a significant signal magnification and immune cell influx to establish

anti-Ag immunity. MF59 became the first emulsion-based VADS approved for delivering the seasonal influenza vaccine of Fluad® for human immunization in 1997 and followed by AS03, which is a 200 nm-sized O/W emulsion consisting of squalene/DL-a-tocopherol/Tween 80 and was approved for human use in GSK's A/H1N1 pandemic flu vaccine Pandemrix® [33]; and then AF03, which is a 80 nm-sized O/W emulsion consisting of squalene/polyoxyethylene cetyl-stearyl ether/sorbitan oleate/mannitol and was approved for clinical immunization in Sanofi Pasteur's pandemic influenza vaccine, Humenza® [34].

Now, novel types of emulsions are still actively formulated using various functional materials to constitute a VADS possessing desired properties, including high potent immunogenicity, targeting delivery of vaccines toward draining lymph nodes (dLNs) and APCs, enhanced cellular uptake, controlled release of Ags, rendering vaccine lysosome escape, and directing immunoresponses toward the Th1/Th2 type biased or balanced pathway [32]. Meanwhile, attempts in pushing into clinical trials of emulsion VADSs for delivery of cancer vaccines and other applications have also increasingly continued and are accompanied by endeavors in shedding light on the mechanisms involved in the action of emulsion adjuvants. Recently, Schmidt et al. using squalane as an O and distearoylphosphoethanolamine (DSPE) as an emulsifier engineered the TLR3a poly(I:C)-entrapping cationic nanoemulsions with a size of 200 nm and demonstrated that when given to mice the cationic nanoemulsions drained rapidly to the LNs and activated cross-presenting DCs, MPs as well as B cells, resulting in strong Ag-specific CD8+ T-cell responses [35]. The results suggest the squalane-based cationic nanoemulsions may be a promising VADS with the ability to induce strong CTL responses, offering an alternative way to make vaccines against pathogens that can hardly be protected without activated CTLs. Interestingly, using squalene as O but the 100 nm-sized poly(D, L-lactic-*co*-glycolide) (PLGA) NPs as a stabilizer, Ma and coworkers formulated 2 μm-sized Pickering emulsions as a VADS, which retained the force-dependent deformability and lateral mobility of loaded Ags [36]. Mouse experiments proved that the Pickering emulsions enhanced the recruitment, Ag uptake, and activation of APCs which initiated robust humoral as well as cellular immunoresponses, which effectively supported mice to survive a lethal challenge of influenza virus. The outcomes hint that the pliability of vaccine carriers and lateral mobility of Ags may well count in triggering immune reactions and, as such, may well be taken into account when developing certain types of VADS.

In summary, as one of a few types of VADSs that have been approved for human use, certain types of emulsions prove by numerous clinical and preclinical evaluations capable of eliciting strong humoral and/or cellular immunity against heterologous pathogens meanwhile maintain an excellent safety profile, depending on the components as well as the structural characteristics of this fluid carrier. Further development of emulsion VADSs may focus on elucidating the mechanisms underlying the immunopotentiating functions in regard of particularly the relationship between emulsion efficacy, systematic characteristics, and molecular structure of squalene, squalane or other unidentified active materials [32]. Further efforts may well be committed to improving the stability of emulsions to construct a VADS allowing the products to be distributed, at least for some time, out of the cold chain [37], thus facilitating global vaccination against various infections in, especially, some low-income countries or districts, where integral cold chain may not be available.

2.3. VADS constructed with liposomes formed by self-assembly of phospholipids

Liposomes are the phospholipid bilayer-enclosed vesicles and have attracted many research interests in the development of drug delivery system (DDS) as well as VADS ever since its discovery by Bangham et al. in the early 1960s [38]. Liposomes usually consist of one or more concentric lipid bilayers alternating with aqueous spaces [39, 40], with the components of one, or more type of amphiphilic phospholipids such as phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylglycerol (PG), and sphingomyelin (SM), which though form the frame structure of liposomes and are often supplemented with ingredients, such as cholesterol (CHO) and other charged lipids such as stearylamine (SA), N[1-(2,3-dioleyloxy) propyl]-N,N,N-triethylammonium (DOTMA), 1,2-dioleyloxy-3-(trimethylammonium propane) (DOTAP), and 3 (N,N,-dimethylaminoethane)-carbamyl cholesterol (DMACHO), which are purposely used for tailoring the property of liposomes. Depending on ambient temperature and the nature of the lipids, the liposome bilayers may exist in either a "fluid" state when the ambient temperature is above the Tc (a gel to liquid crystalline transition temperature—the temperature at which the acyl chains melt) of liposomes, or a "rigid" state when the ambient temperature falls below the Tc of liposomes [40]. However, when CHO is homogenously incorporated into phospholipid bilayers, for example, at the mole ratio of CHO/PC between 1/3 and 2/3, the membrane rigidity may be significantly strengthened, and as a consequence, liposomes are blurred of Tc lowering content leakage. Bearing a common weakness of instability associated with a colloidal system, liposomes are often superficially PEGylated (modification with polyethylene glycol, PEG) to engender a steric stabilization effect, and/or are charged with an appropriate zeta potential value by the incorporated ionic lipids to generate an electrostatic repulsion for preventing aggregation, or for flocculating particles according to DLVO theory. Also, lyophilization is often employed to engender liposomes into a dry entity, which has a high stability completely satisfying the shelf-life requirements for clinical application, as it can be rehydrated to reconstitute the initial vesicles with little cargo leakage just in the presence of disaccharide as an effective lyoprotectant [41].

Liposomes possess numerous distinct properties enabling them to fulfill the functions of an excellent VADS, which can be summarized as aspects including good biocompatibility, high loading capacity for various ingredients, and the ease for preparation and surface decoration to engender specific functions such as targeting delivery, lysosome escape and controlled release [42]. With ability to entrap hydrophilic, lipophilic as well as amphiphilic molecules in the inner aqueous phase or lipid bilayers, liposomes have been formulated for delivering a large range of therapeutic agents, including small molecules, DNA/RNA fragments, peptides, and even proteins with a large molecular weight (MW), which exhibit respective therapeutic activities [39]. Also, liposomes are frequently employed as a VADS fitting diverse immunization routes, including intravenous, intramuscular, subcutaneous, intranasal, oral uptake, pulmonary inhalation, and topical skin or mucosal administration, for delivering vaccines to resolve problems associated with free Ags, for instances, averting premature inactivation caused by environmental chemicals, and ensuring Ags to approach APCs and even the intracellular organelles without off-targets [43]. In particular, beneficial for acting as a VADS, liposomes possess the intrinsic adjuvant properties as established by Gregoriadis and coworkers in as early as 1974 when strong humoral immune responses to liposome-entrapped

diphtheria toxoid were observed after injection of the liposome vaccines into mice [40, 44, 45]. It is generally accepted that liposomes as a VADS function with the adjuvanticity regardless of the carrying mode of Ags, including entrapment within vesicles, attachment on surfaces, or simply mixing together [46, 47], which allows diverse modifications to be carried out on liposomes without concerning the Ag damage through measures, including PEGylation, decoration with PAMP molecules or the pattern recognition receptor agonists (PRRas), such as lipid A for TLR4, CpG-ODN for TLR9, and synthetic mannose derivatives for C-type receptors on APCs [8, 9, 37, 48, 49].

Notably, multifunctional liposomes have also been developed in combination with novel administration devices to form a VADS which can be employed to enhance immunization efficacy via convenient administration [47]. In particular, Wang's group developed the multifunctional liposome-based VADSs through fabrication of liposomes adorned with TLR4a lipid A and loaded with Ags into biodegradable microneedle arrays, which can efficiently exert penetration of mucosa enhancing topical delivery efficiency [37, 48, 50]. Going further, Wang and coworkers engineered two types of multifunctional liposomes, the 200 nm-sized mannosylated lipid A-liposomes (MLLs) and the 50 nm stealth lipid A-liposomes (SLLs), both of which were loaded with Ags and NH_4HCO_3 and then packed together into microneedles, forming the proSLL/MLL-constituted microneedle array (proSMMA), which proved able to rapidly recover the initial MLLs and SLLs upon rehydration by tissue fluids [47]. Mice vaccinated with proSMMAs by vaginal mucosa patching established robust Ag-specific humoral and cellular immunity at both systemic and mucosal systems, especially, in the reproductive and intestinal ducts, owing to the action processes involving the facts that the MLLs reconstituted from the administered proSMMAs were mostly taken up by vaccination site-resident DCs for mucosal responses, whereas the smaller SLLs traveled to the dLDs wherein picked up by macrophages for efficient use of Ags. Furthermore, the delivered Ags were displayed by APCs via cross-presented with MHC-I thanks to lysosome escape and ROS (reactive oxygen species) stimulation, which were caused, respectively, by expansion of CO_2 gas and induction of excessive NH_4^+/NH_3 both sourcing from the liposome-released NH_4HCO_3, leading to a mixed Th1/Th2 type response promoted further by liposomal lipid A and activation of TLR4. Thus, though the large-scale production of the proSMMAs seems still a problem owing to the complex procedure for products and the instable entrapment of volatile NH_4HCO_3 in vesicles, the multifunctional VADS constructed with liposomal microneedles for vaginal immunization provides an alternative strategy to elicit immunity against various pathogens, especially, the sexually transmitted ones. Moon et al. fabricated a novel VADS based on a special type of liposomes, which were called interbilayer-crosslinked multilamellar vesicles (ICMVs) and formed by crosslinking headgroups of adjacent phospholipid bilayers within multilamellar vesicles [51]. Further investigation showed that the stable Ag/adjuvant-carried ICMVs rapidly released the loaded cargos in response to catalysis by endolysosomal lipases, and when given to mice elicited robust endogenous T-cell and antibody responses, suggesting ICMVs a stimulus-sensitive VADS which may open up new possibilities for vaccination against infectious diseases and cancer.

Summarily, liposomes are the most diverse carrier for delivering various agents and can be employed through diverse modifications with various functional molecules to constitute

different types of multifunctional VADS satisfying different vaccination requirements. As a proved by numerous experiments, at least in animal models, these multifunctional liposome VADSs are highly effective in both targeting delivery of vaccine to APCs and enhancing Ag presentation by APCs to related T-cells to set up the Ag-specific immunity against pathogens, fulfilling a dual function of adjuvancy and delivery for vaccines [7, 37, 47–49].

2.4. VADS constructed with ISCOMs formed by self-assembly of saponin and lipids

The immune stimulating complexes, named ISCOMs, are a type cage-like NPs with a size of 40 nm constructed of linked nanoring subunits with a size of 12 nm, and usually formed through self-assembly of the main components of phospholipids, cholesterol and, importantly, saponin which, as a crude mixture of numerous triterpene derivatives extracted from the cortex of the South-American Tree *Quillaja saponaria Molina*, has potent adjuvant activities [52]. ISCOM was first coined the name in 1984 by Morein et al. [53], who demonstrated that ISCOMs contained saponin Quil A, a heterogeneous mixture containing up to 23 different saponin compounds [54], and virus membrane proteins were at least 10 times more potent than micelles formed by aggregation of the protein Ags alone, but caused no severe side effects, such as hemolysis, associated with saponin. The strong immunostimulatory effects were argued to be resulted from large exposure of protein Ags in ISCOMs and the intrinsic adjuvanticity of saponin Quil A, while no severe adverse effects of hemolysis associated with saponin were noticed thanks to its tight association with cholesterol.

Being explored for high potency and low toxicity, Quil A was purified using reversed phase high performance liquid chromatography (RP–HPLC), by which Kensil et al. identified adjuvant activity in 10 of the fractions including the four most abundant compounds, termed QS-7, 17, 18, and 21, with the numbers corresponding to their relative elution time, which is dependent on their degree of hydrophobicity using C4 resin column with RP–HPLC [55]. Similarly, Rönnberg et al. isolated three different RP–HPLC fractions of Quil A: QH-A sequences eluted early, further two sequences of the more hydrophobic fractions QH-B and QH-C, which were examined by pre-clinical toxicology and animal testing, resulting in an optimized combination of 7 parts QH-A, 0 parts QH-B and 3 parts QH-C, known as QH-703 or ISCOPREP™703 (Iscotec AB, Sweden) [56], which was further developed into proprietary product ISCOPREP™ saponin by omitting QH-A fraction [57].

The identification of purified adjuvants from crude saponin allows ISCOMs to be formulated with more defined ingredients, such as monomer of QS21, ISCOPREP™ 703, and ISCOPREP™ [54], to constitute a VADS which can induce robust immunoresponses with Ags whether incorporated in the carrier or just physically mixed with the carrier [58, 59]. Formulation requiring no Ag incorporation not only simplifies the process of preparing the ISCOM vaccines but also expands the delivered Ags to include the hydrophilic ones; and the findings further supports the hypothesis that encapsulation of Ags in a carrier is not necessarily the prerequisite requirements for stimulating immunoresponses [60]. Duewell et al. developed the palmitified OVA-incorporated ISCOMs consisting of ISCOPREP, PC and cholesterol and showed that subcutaneous injection of OVA-ISCOMs to mice resulted in a substantial influx and activation of immune effector cells in dLNs in control of the vaccinated site and promoted natural killer (NK) and NK T cells to produce IFN-γ. Also, facilitated by the efficient Ag cross-presentation CD8α^+ DCs in dLNs, a high frequency of different tumor cell killing Ag-specific CTLs was

differentiated and proliferated from relevant precursors [61] through MyD88 (the myeloid differentiation primary response gene 88) adapter protein-expression pathway, as revealed by Wilson et al. [62]. Notably, ISCOMs were upgraded by Schiött and coworkers to the next generation VADS, denoted Posintro™, which were cationic NPs formulated with cholesterol, DC-cholesterol (3β-(N-(N′,N′-dimethylaminoethane)-carbamoyl) cholesterol hydrochloride), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), Quil A and HBsAg in the weight ratio of 3:1:4:20:5, engendering a new HBV vaccine of Posintro™-HBsAg [63]. In the intradermally (i.d.) immunized animal models of mice and guinea pigs, Posintro™-HBsAg induced the strong response with high titers of HBsAg-specific antibody and high levels of cytotoxic T lymphocyte (CTL), demonstrating that Posintro™-HBsAg is promising both for the protection against HBV infection and as a potential therapeutic vaccine.

Notably, to develop effective vaccines against the deadly Ebola virus (EBV), which causes a fatal hemorrhagic fever in humans with a mortality of around 50%, as evidenced by the 2014–2016 West Africa Ebola epidemic which claimed 11,310 lives in 28,600 infection cases [64], Bengtsson et al. engineered the 2014 EBV Makona strain glycoprotein (EBV/Mak GP) trimer VLPs (virus-like particles) with a size of 30–40 nm using the Sf9 (engineered *Spodoptera frugiperda*) insect cell-recombinant baculovirus expression system [65]. In mice, adjuvanted with the Matrix-M which consists of two populations of 40 nm ISCOMs: 85% Matrix-A of saponin QH-A fraction +15% Matrix-C of saponin QH-C, EBV/Mak GP VLPs induced a rapid onset of specific IgG and neutralizing antibodies, increased frequency of multifunctional CD4+ and CD8+ T cells as well as effector B cells. Noteworthy, the immunity established in the vaccinated mice conferred a 100% protection against a lethal viral challenge, suggesting the Matrix-M adjuvanted EBV/Mak GP VLP NPs an effective VADS for developing subunit vaccines against the deadly Ebola infections. Similarly, the group using Sf9 insect cell platform engineered a recombinant trivalent NP influenza vaccine (tNIV), which when intramuscularly administered with Matrix-M to ferrets induced high levels of broadly neutralizing antibodies against A (H1N1) strain, B strain and, especially, a panel of all historic (2000–2017) A/H3N2 strains [66]. In particular, in a clinical trial involving 330 adults, the 60-μg dose of tNIV/50 μg Matrix-M induced significantly greater HA inhibition antibody responses against a panel of wild-type A (H3N2) strains than did the inactivated trivalent vaccine Fluzone [67], showing that Matrix-M/tNIV may be an efficient strategy for developing the effective universal influenza vaccines with additional advantage in avoidance of the mismatching Ags as occurred in conventional procedures.

Summarily, the nanosized cage-like ISCOMs constituted through self-assembly of a combination of saponin, phospholipid and cholesterol are a multifunctional VADS, which can deliver or adjuvant Ags and, in both cases, can enormously boost the efficacy of subunit vaccines. In particular, ISCOMs can be combined with other adjuvants such as TLRas to further improve the immunostimulatory effects for enhancing function of adjuvanted Ags, thus providing a diverse platform for making therapeutic as well as prophylactic vaccines against pathogens or malicious neoplasms.

3. Conclusions

The NP-based VADSs provide an efficient strategy for delivering and enhancing efficacy of subunit vaccines, which are weak immunogens but represent the current trends in the

development of vaccines against various pathogens including cancer. The NPs formed through self-assembly of small molecules, especially, those possessing intrinsic adjuvanticity, are an attractive and promising VADS due to their numerous advantages, such as acceptable safety profile, ease for preparation and modification with functional materials as well as control of size, and fitting different vaccination routes, which may confer the carried vaccines multiple functions capable of eliciting the Ag-specific humoral as well as cellular immunity at both systemic and mucosal levels providing a strong protection against pathogens. Encouragingly, some subunit vaccines developed with the VADSs that are based on small molecule-assembled NPs have already been approved for clinical vaccination, and typical products include the virosome-based hepatitis A vaccine (Epaxal®) and influenza vaccine (Inflexal V®), MF59-based influenza vaccine (Fluad®), AS04-based HPV (Cervarix®) and HBV (Fendrix®) vaccines, and AS01-based malaria vaccine (Mosquirix®). Hopefully, as many problems associated with NP VADSs, such as high cost for products, and undefined mechanisms underlying immune reactions and associated adverse effects, are finally settled, more NP VADS-based subunit vaccines will be pushed into markets for conquering human life-threatening diseases, such as HIV infection, MERS infection, and even intractable cancers.

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Conflict of interest

All the authors declared no conflict of interest.

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Chapter 3

Polymeric Nanoparticles Engineered as a Vaccine Adjuvant-Delivery System

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Additional information is available at the end of the chapter

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Abstract

Global immunization saves millions of human lives each year through using vaccines, which include whole microbe-based products and the subunit ones formulated with just the components of antigens able to stimulate immune system to establish specific immunity against diseases. Subunit vaccines show numerous advantages, such as defined components, high safety profile, and production without the use of dangerous pathogens, but also limited capacity in eliciting immunity due to the lack of other components than antigens, including the immunostimulatory elements of pathogen-associated molecular patterns which are able to activate the innate immunoreponses. Recently, nanoparticles (NPs) formulated with polymeric materials, such as poly(lactic-co-glycolic acid), viral proteins, chitosan, hyaluronic acid, and polystyrene, with some bearing intrinsic adjuvanticity, are widely employed as vaccine adjuvant-delivery systems (VADSs) and show great potential in developing subunit vaccines. Particularly, the polymeric NPs engineered with functional materials possess many features, such as targeting delivery, lysosome escape, anti-damaging protection, and ability to guide immune reactions toward a Th1 (T helper type 1) and Th2 pathway, which are crucial for establishing humoral and cellular immunity. This chapter describes polymeric NP-based VADSs designed for developing subunit vaccines able to elicit Ag-specific immunity at both systemic and mucosal levels via different vaccination routes.

Keywords: polymer, nanocarrier, immune response, mucosal vaccination, cellular immunity, pathogen/danger-associated molecular pattern (PAMP/DAMP), targeting delivery

1. Introduction

Vaccines have saved countless human lives from lethal infections since the modern use of vaccinia against smallpox by British physician Edward Jenner in the late eighteenth century and are today playing more and more crucial roles in fighting life-threatening diseases, of which, due to great advances made in branches of the field and related fields such as immunology and biotechnology, the scope has enormously expanded, ranging from the earlier aim of microorganism infections to the novel targets of autoimmune disorders, allergic reactions, and even malicious cancers [1]. However, the list of infectious diseases for which vaccines are urgently needed but do not yet exist is till long and in particular many pathogens such as HIV (human immunodeficiency virus), HSV (herpes simplex virus), and HCV (hepatitis C virus), showing on their surface elusive or ever changing immunogenic features to continuously dismantle a variety of potential arsenals; let alone numerous malignant tumors, autoimmune disorders such as multiple sclerosis, diabetes, and rheumatoid arthritis, which are all aggressively threatening human health and life, posing a big challenge to developing effective vaccines or immunotherapy [2].

Vaccines function against diseases through stimulating the host immune system with the antigenic components featured by pathogens or neoplasms to establish the antigen-specific immunity which is able to clear the abnormalities bearing the identical antigens. Vaccines developed for handling infectious diseases are mostly manufactured using the live attenuated or killed whole microbes, which have a high potency in triggering immune system but are argued to be associated with possible reversion to virulence due to mutation of administered strains, as evidenced by gene sequencing in vaccinated sufferers, thus causing safety concern. As many mechanisms underlying immunoresponses are revealed and great achievements are made in relevant fields, subunit vaccines, which are formulated with defined components including Ags to induce immunoresponses accurately targeting the matched objects thus causing few safety concerns, are now more and more employed to fight not only infectious pathogens but also other illnesses, providing, in particular, outstanding ways to overcome the previously intractable diseases, such as cancer [3]. Compared to conventional whole microbe vaccines, subunit vaccines possess many distinct properties which are beneficial for clinical applications, summarily including high safety profile, production needing no dangerous microorganisms, no redundant components to cause allergic or autoimmune responses, diverse usage including anticancer, and high capacity for several peptide epitopes targeting different stages in the life cycle or subtypes of a pathogen [4].

However, subunit vaccines are often weak immunostimulatory products, due to lack of such components as the pathogen-associated molecular patterns (PAMPs), which are expressed on a microbe surfaces and are able to activate the pattern recognition receptors (PRRs), such as the TLRs (toll-like receptors), NOD-like receptors (the nucleotide-binding oligomerization domain-like receptors), RIG-I-like receptors (retinoic-acid-inducible gene-I-like receptor), and C-type lectin receptors, thus facilitating host immunoresponses [5]. As such, subunit vaccines are often formulated with an adjuvant, which is a nonspecific immunopotentiating substance able to elevate, either in advance or simultaneously with the vaccine Ags, the immune

response of recipients to the Ags, or change the type of immune responses; otherwise, subunit vaccines are engineered with functional carriers to form a vaccine adjuvant-delivery system (VADS), which is frequently made of a range of NPs (nanoparticles) using various materials capable of targeting the professional Ag-presenting cells (APCs) such as dendritic cells (DCs) and macrophages (MPs) to boost enormously the immunostimulatory activity of a vaccine and is thus making full use of Ags [6–10].

This chapter elaborates the material basis, formulation, rationale, and the state-of-the-art advances in the development of a VADS constructed with polymeric NPs which are made of certain crucial types of polymers such as PLGA, HA, polystyrene, or VLPs, which possess many beneficial features for eliciting immunity against a range of diseases. Thus, this comprehensive introduction will provide a useful reference to interested readers who may thus be attracted to denote their innovative talents to the development of vaccines based on VADS constructed, probably, with polymers.

2. Different polymeric NPs designed as a VADS

Polymeric NPs, namely NPs made of highly biocompatible polymers, such as polystyrene, PLGA, proteins, chitosan, and hyaluronic acid (HA), have recently been widely explored as a DDS (drug delivery system) as well as VADS and show many excellent properties beneficial for therapeutic delivery of agents, for example, high stability can shelter the loaded Ags from environmental detriment and in vivo unwanted degradation; biocompatibility can reduce toxicity to recipients and their compliance; ease in modulation of particle size, surface charge, and specific binding characteristics allow developing multifunctional VADS [3, 4]. Thus, through fulfilling multiple functions, polymeric NPs are able to improve the efficacy of vaccines, for example, they can form a depot to enhance vaccine efficacy via elongated release and exposure of Ag at the site of injection; they can targetedly deliver vaccines to APCs promoting cellular uptake of Ags and thus enhancing Ag stimulation efficiency; they can alter intracellular process of Ags adapting immune responses toward the beneficial humoral and/or cellular pathways; and also they can provide diverse administration routes for vaccination to elicit the desired immunity at circulation system as well as mucosal sites [6, 11].

2.1. Polystyrene NPs as a VADS

Although polymers suitable for constructing VADSs are usually thought to be biodegradable one since they cause no size-limited excretion and associated toxicity concern, certain nonbiodegradable materials possessing certain specific properties, such as chemical inertness and ease for fabricating stable NPs, are also the preferred candidates by researchers for engineering the kind of NPs with an accurate size and special shape, so as to be employed reliably to investigate these physical properties on the immune system and immune responses. For example, Plebanski and coworkers using nonbiodegradable polystyrene NPs performed studies on VADS and showed that polystyrene NPs loaded with OVA epitopes induced different immune responses in a size-dependent manner needing no additional adjuvant, and

that among different particles with a size ranging from 20 to 2000 nm, 40 nm NPs induced the strongest cellular and humoral immunity [12]. Further investigations demonstrated that covalent linkage of peptide to NPs is a requirement for eliciting immunization efficacy and also proved that 40-nm-sized NPs serve as a VADS owing to their preferential uptake by APCs and their ability to traffic to lymph nodes to induce strong immune responses compared to their larger counterparts [4, 13].

Notably, Schöttler et al. recently reported the counterintuitive research results on cellular uptake of NPs with PEGylation (modification with polyethylene glycol), which is gold standard in removal of immune clearance of in vivo NPs through the mechanism of reducing nonspecific cellular uptake of nanocarriers [14]. The researchers documented that polystyrene NPs, which had been modified with PEG or poly(ethyl ethylene phosphate) (PEEP), only had been exposed to plasma proteins, could exhibited a lowered cellular uptake by macrophages (RAW264.7 cells), whereas those not exposed to plasma proteins showed high nonspecific uptake. Further mass spectrometric analysis revealed that the plasma-exposed nanocarriers formed a protein corona which was identified to contain just an abundance of clusterin proteins (known as apolipoprotein J) and to be the decisive factor controlling lowered nonspecific cellular uptake of the PEGylated or PEEPylated polystyrene NPs, and to contrast, the classic conception that PEGylated NPs free of immune clearance is resulted from avoidance of protein adsorption. These outcomes indicated that PEG as well as PEEP can affect the composition of protein adsorption by polystyrene NPs, and that the presence of certain type proteins may be just a prerequisite in preventing nonspecific cellular uptake of NPs, defying the conventional belief that PEGylation reduces protein adsorption thereby conferring a stealth effect [15].

2.2. Virus-like particles as a VADS

Virus-like particles (VLPs) consist mainly of viral proteins devoid of viral genomes to mimic the natural structure of virions and have been engineered to carry agents for various applications, including, particularly, for constructing a VADS for delivering subunit vaccines based on their viral envelop structures suitable for presenting functional spikes on NP surfaces to maintain the intrinsic immunogenicity apt to trigger immunoresponses [16]. VLPs are usually manufactured using protein expression systems based on bioengineered bacteria, yeast, insect, avian, mammalian or plant cells, or using cell-free protein synthesis system (CFPS), which provide an alternative to construct effective VADSs with beneficial characteristics, such as having defined structure formed through self-assembling, large cargo loading capacity, easy functionalization with ligands, and high stability and low toxicity [17]. As a VADS for producing vaccine candidates, VLPs are often designed with characteristics identical to native virus especially in the aspects of the immunochemical properties, 3-D (3-dimensional) architectures and morphological conformations, through engineering on their particulate structures, which, like the native virus, include the nonenveloped and enveloped types. The nonenveloped VLPs mainly consist of one or more pathogenic components, but do not contain any components of the expression hosts, while the enveloped VLPs generally consist of matrix proteins which are enveloped in a lipid membrane derived from the expression hosts, possibly, with glycoproteins embedded into the bilayered membranes.

VLPs are widely used as a VADS because they possess several clear advantages, including induction of immunity with a broad cross-protection rendering onetime immunization to

protect against different virus genotypes, high potency to trigger immune responses providing an option to conquering intractable pathogens such as HIV and HCV (hepatitis C virus), high thermostability possibly requiring no integral cold chain to keep viability favoring global vaccination, and high manufacturing efficiency in large scale while at low cost offering a strategy to handle the emergency arising from infectious diseases, such as Ebola outbreak and epidemic. For example, HCV infection is still a significant public health problem, though it has been partially addressed with the advent of directly acting antiviral agents (DAAs), which represent a major advance toward controlling HCV but confer little protection against reinfection [18]. Presently, around 71 million people in the world are living with chronic HCV infection, and each year nearly half a million of them will die of HCV infection or its complications, rendering it urgent to develop an effective vaccine capable of eradicating HCV, which may well be produced using the VLP-based VADS, in reference to HBV vaccines. Recently, a quadrivalent genotype 1a/1b/2a/3a HCV VLP vaccine was successfully engineered by researchers using scale-up production methods of Huh7 cell factories containing a recombinant adenoviral expression system representing each HCV genotype, followed by cell lysing and purification with iodixanol ultracentrifugation and stirred cell ultrafiltration [19]. When given subcutaneously to mice, whether in the presence of an adjuvant (system) or not, the quadrivalent vaccine consistently induced production of Ab and nAb (neutralizing Ab) together with robust T and B cell responses for eliciting broad humoral and cellular immunity, indicating the VLP-based VADS a useful tool which may be employed for the production of an effective HCV vaccine [20].

Globally, HIV continues to be a major public health issue and also claims approximately 1 million lives each year, although total people living with HIV have the opportunity to receive antiretroviral drugs (ARVs), which may effectively control the virus from transmission and causing illness but are also found to be undermined in efficacy by pathogens that had evolved with drug resistance [21]. Still, a highly effective vaccine is believed to be the ultimate weapon able to erase HIV and associated disease AIDS (acquired immune deficiency syndrome), though at present, there are no such a product in markets and, moreover, many products developed in previous years bearing such expectations failed to show clinical efficacy in fighting this rapidly mutating pathogen, including especially the big trial, known as STEP, which was halted in 2007 after the vaccine was found to increase the risk, instead of prophylaxis, of HIV infection [22]. Nevertheless, scientists are getting closer than ever to developing such an effective product, as evidenced by a large-scale clinical study conducted in 2009 in Thailand (called RV144) showing that immunization with a combination of two HIV vaccines prevented about 31% of new infections through a prime-boost combination regime [23], which comprises four priming intramuscular injections of ALVAC-HIV, which is a recombinant canarypox vaccine express HIV-1 Gag, Pro and gp120-gp41, plus two boosting intramuscular injections of AIDSVAX® B/E, which is an alum-adjuvanted bivalent HIV-1 gp120 vaccine of subtypes E and B [24]. Though the low prevention rate of vaccination with ALVAC/AIDSVAX combination excludes the products from approval for clinical prophylaxis of HIV, the moderate effects displayed in human trial not only provoked scientists to make deep explorations on the causes for the failures, but also presented researchers a great encouragement to commit further efforts to developing efficacious HIV vaccines. Subsequently, based on the virion features which are more and more clearly elaborated in structure and function, scientists set out to handle the obstacles identified to the development of a preventive HIV vaccine from several aspects, such as accurately targeting the conserved antigenic proteins, seeking Ags able

to induce the broadly neutralizing Abs (bnAbs), and formulating a highly efficient VADS [25]. As mentioned above, a VLP-based VADS proves a highly potent inducer for Abs and helper T cell responses and also able to elicit robust cytotoxic T cell responses necessary for preventing primary infections and erasing infected cells, thus offering researchers an alternative tool to engineer effective vaccines against HIV, which is regarded as the most challenging foe owing to its poor immunogenicity, fragile surface glycoprotein, and the ability to overpower the cell immune system [26].

Recently, Chapman et al. constructed an MVA (modified vaccinia Ankara)-mGag (an HIV-1 subtype C mosaic Gag immunogen) and a DNA-mGag vaccine, which were designed to address the tremendous diversity of HIV, and showed that mGag budded from cells infected and transfected with MVA-mGag and DNA-mGag, respectively, formed VLPs [27]. In mice, the DNA-mGag homologous prime boost vaccination elicited predominantly CD8+ T cells, and the homologous MVA-mGag vaccination induced predominantly CD4+ T cells; in contrast, a heterologous DNA-mGag prime MVA-mGag boost induced strong, more balanced Gag CD8+ and CD4+ T cell responses that were predominantly of an effector memory phenotype. Also, it was found that DNA-mGag homologous vaccination induced much higher cumulative Ag-specific IFN-γ secretion responses and generated significant higher levels of cytokine-positive CD8+ T cells than DNA-nGag (natural Gag), indicating a heterologous prime-boost regimen with DNA and MVA vaccines expressing HIV-1 subtype C mosaic Gag as an Ag is highly immunogenic and may be an effective VLP-based VADS for eliciting strong immunity to HIV. Yao's group formulated a VLP-based HIV vaccine, which was composed of HIV_{IIIB} Gag and HIV_{BaL} gp120/gp41 envelope as a pseudovirion vaccine capable of presenting Ags in their native conformations and was engineered through using HEK (human embryonic kidney cell)-derived cell line expression system [28]. The researchers demonstrated that mice vaccinated by intranasal prime followed by two sub-cheek boosts with VLPs adjuvanted with liposomes entrapping TLR3 ligand dsRNA were stimulated to secrete high titers of Abs against the Ags, with predominant IgG2c over IgG and produce a significantly increased germinal center B cells and T follicular cells, suggesting that the VLP-based VADS is superior for induction of a Th1-biased immune response, while prolonging lymph node germinal centers, T follicular cells, and generating neutralizing antibodies, and thus is rather suitable for making HIV vaccines [26].

Notably, certain types of pathogens that are once known to cause only a mild and self-healing illness and therefore never listed in dangerous items and may abruptly cause the unexpected problems associated with human and population health, hinting the existence of undisclosed infection mechanisms and pathophysiological processes or the emergence of mutations relevant to severe toxicity. For example, during the 2015–2016 South American Zika epidemic, the mosquito-borne virus which used to cause mild symptoms, such as fever, skin rash, and joint or muscle pain, was eventually identified able to cause severe damage to fetal brain through infecting pregnant women and thus finally recognized as the culprit responsible for thousands of microcephaly affected new borns, raising a great social problem and concern [29]. Unfortunately, up to now, still there are no licensed vaccines for prophylaxis of Zika, though several conventional approaches have been tried on developing such as an urgently needed products, including inactivated, recombinant live-attenuated viruses, protein subunit vaccines, RNA and DNA vaccines, as well as the VLP-based VADS [30]. Recently, using

HEK293 cell (Human embryonic kidney 293 cell) expression system, Salvo et al. engineered a VLP-based VADS composed of Zika prM/E (pre-membrane and envelope) glycoproteins for making vaccines to defend against Zika and demonstrated that mice injected with Zika VLP combined with adjuvant alum secreted high levels of the Ag-neutralizing Abs [31]. In particular, the vaccinated mice all survived without morbidity or weight loss after receiving the lethal challenge with the dose of 200 PFU of Zika strain H/PF/2013, proving the protective efficacy of the VLP-based Zika vaccine which may be tested in humans as a prophylactic candidate with minimal safety concerns to protect unborn babies whose mothers become infected with Zika during pregnancy.

Similarly, Espinosa and colleagues formulated a ZIKV vaccine based on virus-like particles (VLPs) which were generated in HEK293 cells transiently transfected with the prM/E genes of Zika placed downstream from a heterologous signal sequence and observed efficient induction of neutralizing antibody and a dose-sparing effect of alum in VLP-immunized mice (C57Bl/6 x Balb/c) [32]. In addition, passive transfer experiments showed that AG129 mice received the sera from immunized mice prior to Zika infection manifested significantly reduced viral replication as indicated by viral RNA levels in the blood and successfully conquered the infection to contrast control mice which succumbed to infection, underscoring the protective effect of the humoral immunity elicited by this VLP-based Zika vaccine candidate.

In summary, the VLP-based VADSs are a potent inducer of Ab and cellular responses and also possesses the prerequisite features required to prepare the vaccines that are able not only to prevent the primary infections but also to clear infected cells, thus representing an alternative tool promising to engineer efficacious vaccines against the intractable pathogens, such as HCV, HIV, and even parasites [33].

2.3. Chitosan NPs

Chitosan, a linear polysaccharide composed of randomly distributed β-(1,4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit), is usually made through hydrolysis of the chitin shells of shrimp and other crustaceans with an alkaline substance, such as sodium hydroxide [34]. Chitosan has a positive charge under neutral conditions due to protonation of basic amine groups, which contributes to the enhancement of solubility of the compound; however, the dissolution behavior of chitosan in aqueous media is also negatively influenced by the number and structural form of acetylated groups, allowing chitosan able to be used for agent delivery in several distinct forms, including solution, hydrogel, and especially nano/microparticle, which can be obtained via crosslinking, ionotropic gelation or precipitation-coacervation [35]. Interestingly, novel chitosan derivatives with customized biochemical properties are still continuously synthesized through facile conjugation of side chain moieties of functional molecules to solvent-accessible amine and hydroxyl groups, rapidly expanding chitosan in application range and dosage form [36].

In retrospect, in 1980s, researchers observed that chitosan of 70% deacetylated chitin could activate peritoneal macrophages [37] and induce production of various cytokines in mice [38], which was subsequently further explored by Illum et al. to demonstrate that in mouse model chitosan, via nasal immunization, could act as an efficient VADS able to remarkably enhance

local as well as systemic Ab responses toward the vaccines containing filamentous hemagglutinin from *Bordetella pertussis* [39]. The researchers also proved that chitosan could significantly elevate immunogenicity of the nasal vaccine of diphtheria toxoid (DT) and enhanced vaccines in induction of high levels of Ag-specific IgG, secretory IgA, toxin-neutralizing Abs, and T cell responses, predominately of Th2 subtype [40].

Chitosan, as a cationic polysaccharide bearing lots of reactive groups, possesses beneficial properties for vaccine formulation, including biocompatibility, flexibility in terms of formulation and degree of deacetylation, and efficacy when administered via mucosal route, and ability to promote immune responses [41, 42], and thus is thought a superior alternative to alum which only favors promoting humoral responses [43]. Also, chitosan is thought suitable for constituting a mucosal VADS thanks to its bio-adhesive character and intrinsic adjuvanticity, which may arise from chitosan-mediated inflammasome activation [44], or from triggering certain type of PRRs such as TLR4 on immune cells [45], in consistence with its parent molecule chitin which is confirmed able to activate immunocytes via binding to mannose receptors and TLR2 to initiate innate immune responses [46]. However, in spite of numerous research outcomes confirming its strong immunostimulatory potency, the exact mechanism underlying the intrinsic adjuvanticity of soluble chitosan remains yet elusive and needs to be fully discovered through deep exploration. More recently, it was demonstrated that after APC uptake, the intracellular chitosan induced mitochondrial damage, characterized by the generation of mitochondrial ROS and release of endogenous DNA into the cytosol, both of which culminated in the activation of the cytosol DNA sensor cGAS (cyclic-di-GMP-AMP synthase) and subsequent STING (stimulator of IFN gene) pathway, leading to translation of type I IFN and the type I IFN-dependent APC maturation to sponsor cellular immunoresponses [47].

In comparison to free chitosan, chitosan NPs that are design as a VADS capable of enhancing both humoral and cellular immune responses to delivered vaccines are proposed to function relevant to not only chitosan properties but also several aspects associated with NPs, including antigen protection, depot formation, enhanced antigen uptake, and presentation, triggering APCs via different pathways to regulate immune reaction pathways [41]. Recently, Dhakal et al. using the ionic gelation method engineered the chitosan NPs that were loaded with killed swine influenza A H1N2 virus (KIV-CNPs) and demonstrated that the nursery pigs intranasally vaccinated with KIV-CNPs produced high levels of systemic IgG and secretory IgA in nasal mucosa, bronchoalveolar lavage fluids, and lung lysates, which, more importantly, were cross-reactive against homologous (H1N2), heterologous (H1N1), and heterosubtypic (H3N2) influenza A virus strains [48]. Also, the vaccinated pigs demonstrated high frequency of Ag-specific CTLs and lymphocyte proliferation, and stimulation-recalled IFN-γ secretion, leading these pigs to experience reduced severity of macroscopic and microscopic influenza-associated pulmonary lesions after challenge with heterologous viruses, firmly confirming that the NPs composed of chitosan may function as an effective mucosal VADS favoring noninvasive immunization. In another report, Lebre et al. prepared chitosan-aluminum nanoparticles (CH-Al NPs) with a size of 280 nm and a positive surface charge and proved that CH-Al NPs loaded with hepatitis B surface antigen (HBsAg) were more stable in physiological environment and more efficient in inducing cellular immunity than common chitosan NPs, suggesting the two combination immunostimulants chitosan and aluminum salts may

be a promising VADS system for antigen delivery [49]. Interestingly, to develop a vaccine able induce robust mucosal immunity against *Chlamydia trachomatis* (Ct), which is the most common sexually transmitted infection in humans, Rose and cowokers fabricated the PLGA NPs covered with the mucoadhesive chitosan and used as a VADS for delivering recombinant Ct fusion Ag CTH522 [50]. Mice intranasally immunized with the optimized chitosan-coated PLGA NPs containing Ct Ags established potent Ag-specific systemic as well as mucosal immunity, characterized by high levels of anti-CTH522 IgG/IgA Abs in the lungs and the genital tract and high frequency of IFN-γ producing Th1 cells, suggesting that chitosan-coated PLGA NPs may be a promising mucosal VADS for delivering vaccines against sexually transmitted *Chlamydia trachomatis*.

Notably, in a randomized two center phase I clinical trial, an HIV vaccine consisting of HIV-I Clade C-CN54GP140 envelope glycoprotein was administered to HIV negative female volunteers through intramuscular (i.m.) immunization with glucopyranosyl lipid adjuvant (GLA), intranasal (i.n.) immunization with 0.5% chitosan, and intravaginal (i.va.) immunization with an aqueous gel vehicle [51]. The results indicated that, compared to subjects with i.n. or i.va. immunizations, recipients with three i.m. immunizations at the dose of either 20 or 100 μg CN54 gp140 secreted greater systemic and mucosal antibodies, but even in the i.m. immunized subjects, only modest neutralizing responses against closely matched tier 1 clade C virus were triggered; and the i.n. primed subjects were induced the strongest CD4+ T cell response, and, following additional i.m. boosting, were also induced an anamnestic antibody response, suggesting i.n. immunization of HIV vaccines formulated with chitosan may be an effective prime for i.m. boost.

Summarily, due to good tolerability, safety, and, particularly, mucosa-adhesive properties, chitosan and derivatives represent a promising polymer suitable for constructing mucosal VADS to provide great opportunity for developing mucosal vaccines against numerous pathogens which invade hosts through mainly mucosa. However, the available clinical results indicate clearly that to construct an effective vaccine with chitosan to handle the intractable pathogens, such as HIV, further efforts are needed to commit to optimizing formulation, seeking optimal immunization routes, as well as exploring combination with appropriate adjuvants.

2.4. PLGA NPs

PLGA represents one of the most popular polymers for constructing a VADS due to its excellent safety profile, biodegradable properties, ease for processing NPs through double emulsion method, diverse modification to bear functional groups, and also the established use in several marketed products for controlled or targeted delivery of drugs [52]. It is now clear that in vivo PLGA hydrolyzes into metabolite monomers of lactic acid and glycolic acid, both of which are endogenous and easily metabolized by the body via the Krebs cycle, leaving behind little systemic toxicity, allowing wide use of PLGA as a VADS or DDS (drug delivery system). Notably, one of the appealing issues associated with the use of PLGA NPs as a VADS is attributed to the confirmation that, after cellular internalization via fluid phase pinocytosis or clathrin-mediated endocytosis, PLGA NPs may rapidly escape the endolysosomes and carry the loaded cargoes to cytoplasm, avoiding lysosomal degradation into null fragments and thus enhancing vaccine delivery efficiency [53].

To develop an effective VADS, Noormehr et al. fabricated 500-nm-sized PLGA NPs which were covalently conjugated with recombinant Ags Leishmanial CPA (cysteine peptidase A) and CPB, and proved that mice intra-peritoneally immunized with the inhomogeneous Ag-NPs secreted high levels of NO (nitric oxide) by peritoneal MPs and high levels of IFN-γ by splenocytes, which significantly lowered *Leishmania major* burden, suggesting the Ag-conjugated PLGA NPs can be used as a VADS able to deliver vaccines to protect against the tough pathogen of parasites [54]. To investigate the function of multiple adjuvant-combined VADS, Ebrahimian and colleagues formulated the TLR 7/8a resiquimod- or TLR4a MPLA-loaded PLGA NPs which were physically covered with polyethylenimine (PEI) forming PLGA/PEI NPs and then mixed with CpG ODN (cytosine-phosphorothioate-guanine oligodeoxynucleotide) to engender a complexed entity of resiquimod- or MPLA-PLGA NPs/PEI-CpG ODN [55]. Given to BALB/c mice, the multiple adjuvant-constituted PLGA NPs loaded with Ags induced robust and efficient immune responses, as confirmed by evaluation of vivo cytokine (IFN-γ, IL-4, and IL-1β) secretion and antibody (IgG1 and IgG2a) production, demonstrating using a combination of adjuvants in a context-dependent manner may a feasible strategy for engineering a potent PLGA-based VADS. To make subunit vaccines suitable for immunization via skin, which is an attractive but also very challenging immunization site due to the presence of affluent APCs while difficulty of administration, recently, Bouwstra's group fabricated the hyaluronan (HA)-based dissolving microneedles (MNs) entrapped with PLGA NPs which co-encapsulated ovalbumin (OVA) as an Ag and poly(I:C) as an adjuvant for intradermal immunization [56]. Further investigation indicated that the immunogenicity of the PLGA NPs after administration of dissolving MNs was compared with that of hollow MN-delivered PLGA NPs in mice, while immunization with free Ag in dissolving MNs resulted in equally strong immune responses compared to delivery by hollow MNs. However, humoral and cellular immune responses evoked by PLGA NP-loaded dissolving MNs were inferior to those elicited by NPs delivered through a hollow MN, suggesting several critical parameters should be fully evaluated in engineering the PLGA NP-loaded dissolving MNs as an intradermal VADS to avoid unnecessary efforts on the complexed formulations.

At present, still a large fraction of vaccines require a multiple dosing schedule with a 1- to 2-month gap between administrations to guarantee establishing the Ag-specific immunity strong enough to protect recipients, as such, however, engendering a big challenge to worldwide vaccination, especially, in the developing countries, where healthcare workers are not only in shortage but also confronting difficulty in reaching the subjects multiple times to administer booster shots [57]. Conceptually, this challenge may be conquered using a VADS that are constructed with a functional carrier which release vaccine ingredients in pulses with an appropriate time gap between vaccinations, thus simplifying the vaccination schedule to consist of only once injection to exclude additional visits by a healthcare worker. For this, Tzeng et al. engineered a controlled release VADS consisting of bPEI (branched PEI)-modified PLGA microparticles which contained in inner core Ags of IPV (inactivated polio vaccine with three antigens) and an Ag stabilizer poly(L-lysine) [58]. Further investigation indicated that the bPEI-PLGA microparticles stabilized IPV in its active conformation inside the particles for months but in an aqueous medium released two bursts of IPV with an interval of just 1 month, vividly mimicking a typical twice vaccination schedule. Moreover, one injection of the controlled-release formulations elicited a similar or better Ag neutralizing response in rats compared to

multiple injections of liquid vaccine, suggesting the VADS constructed with the bPEI-PLGA microparticles has big potential to elevate vaccine coverage in the developing world.

Conclusively, PLGA is a biodegradable, safe, and clinically used polymer, which, using the double-emulsion method, can be conveniently engineered into NPs to constitute a VADS with appropriate features and abilities to render vaccine lysosome escape, thus enhancing vaccination efficiency.

2.5. Hyaluronic acid (HA)-modified liposomes

Hyaluronic acid (HA) as a polysaccharide consists of alternating units of D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (Glc-NAc), connected to each other with β-1,3- and β-1,4-glycosidic bonds, having nearly perfect chemical repeats except for occasional deacetylated glucosamine residues to form a very hydrophilic linear high molecular weight (HMW) biopolymer [59]. HA ranges in size from 5 kDa to 10 million Da (corresponding to 25,000 disaccharide units), with the most common forms of 1–8 million Da in humans and can absorb water to expand its solid volume by up to 1000 times forming a very viscous and elastic gel [60]. HA GlcA carboxyl group is dissociated at physiological pH values to engender a negatively charged polymer which is readily combining with the most prevalent extracellular cation of Na^+ to form sodium hyaluronate, suggesting that the molecule is not ionized [59]. In fact, while native HA with a high molecular weight (HMW) acts mainly as a constructive stuff and a control on tissue hydrodynamics, low molecular weight (LMW) HA usually participates in cell signaling through interaction with certain types of cell surface receptors, such as CD44 as the primary one, but also TLR2 and TLR4, thus contributing to several physiological and biological activities [61, 62].

As an abundant endogenous polymer, HA has been widely exploited to construct the functional carriers for delivering various bioactives with expectation of improving human health, given many of its desirable merits that can be employed for optimizing delivery effects [63]. Most attractively, HMW HA possesses numerous physicochemical and physiological features, such as biocompatibility, biodegradation, mucoadhesive property, bearing negative charges in a neutral condition, possessing active groups allowing various functional modifications that are all beneficial characters for engineering carriers to deliver agents [64]. Another interesting aspect lies in that LMW HA binds to several receptors, especially CD44, which is ubiquitously expressed on various cells, and especially overexpressed on many types of cancer cells, presenting bases for developing a tumor-targeting drug delivery system (DDS) with attractive advantages, such as the ease of associating drugs with the polysaccharide or its carrier thus solving any solubility problems, improving a drug's blood plasma half-life thus playing a similar role to PEG, and high tumor-targeting efficiency, and as such is currently the main trend in the HA-based delivery research [65]. More recently, LMW HA is focused on its ability to activate CD44 or TLRs on immune cells involving regulation of certain signaling pathways associated with APC maturation, cytokine production and innate immune responses for immunization, and even, in a CD44- and TLR4-independent manner, to enhance CCR7 expression on DCs promoting DC recruitment to tumor regional lymph nodes and restraining DC migration toward tumor tissue [62, 66–68], thus providing not only a comprehensive option for engineering functional nanoparticles fitting a VADS, but also a promising candidate for improving DC maturation in the context of DC-based vaccine development.

Up to now, most of the HA-based carriers used as a VADS have been developed by making use of the "nonbioactive" properties, which has little physiological interference on the body and as such, is used just as constructive stuff. Moon's group formulated the HA-PEG-shelled cationic DOTAP/DOPE liposomes carrying F1-V, a candidate recombinant antigen for *Yersinia pestis*, as a stable and potent nasal VADS, which exhibited markedly decreased cytotoxicity associated with DOTAP liposomes to BMDCs, and when further incorporated with MPLA promoted BMDC maturation and induced a strong Th1/Th2-balanced immunoresponse toward Ags, as evidenced by high titers of F1-V-specific total IgG, IgG1, and IgG2c produced in intranasally immunized mice [69]. Huang's group engineered mLCP (the mannosylated lipid-calcium-phosphate NPs) and LPHa NPs (liposome-protamine-HA-anisamide NPs) for, respectively, targeting delivery of the tumor antigen Trp 2 peptide/CpG ODN to APCs and the TGF-β-silencing siRNA to tumor cells which overexpress sigma receptors with a ligand of anisamide [70]. They demonstrated that the delivery of Trp 2/CpG ODN to DCs by mLCP-based VADS elicited a potent systemic immune response to tumors in mice but generated, to later stage B16F10 melanoma, a marginal efficacy, which however, was remarkably boosted through silencing the immune-suppressive cytokine TGF-β in tumor cells with siRNA-loaded LPHa NPs to engender increased tumor infiltrating CD8+ T cells and decreased regulatory T cells within tumor microenvironment. Wu's group fabricated a microneedle array (MA) with HA with a deep cave formed in the basal portion of each microneedle, into which BCG (Bacille Calmette-Guerin bacillus) powder could be packaged directly, thus producing a painless VADS of MA-BCG, which after vaccination by patching on skin of mice caused no overt skin irritation, but elicited strong humoral and cellular immunity comparable to that of intradermal immunization [71]. Notably, other researchers showed in a clinical trial that HA-constructed MA containing trivalent influenza hemagglutinins (A/California/07/2009 (H1N1), A/Victoria/210/2009 (H3N2), and B/Brisbane/60/2008, 15 μg each) induced immune responses against A/H1N1 and A/H3N2 strains equal to that by subcutaneous injection groups without stirring severe local or systemic adverse reactions and engendered the efficacy against the B strain much stronger than that by the injection group, proving HA-MA a promising practical use as an easy and effective method to replace conventional injection systems [72].

Recently, Hahn's group conjugated an antigenic peptide of myostatin fragment (MstnF) to HA with a LMW (17 kDa) for transdermal vaccination against Duchenne muscular dystrophy (DMD), which is a neuromuscular disorder accompanied with muscle weakness and wasting with myostatin emerging as a key negative regulator [73]. In vivo experiments demonstrated that HA-MstnF conjugates efficiently penetrated into deep skin layers, and HA exerted a boosting effect on the immunization of MstnF in the transdermally vaccinated mice, which not only secreted high levels of antibody titers against myostatin but also showed a significant improvement in the pathological status of skeletal musculature as well as functional behaviors. Gonzalez-Aramundiz et al. prepared protamine/LMW HA (162 kDa) NPs using a mild ionic cross-linking technique and showed that in vitro Ag (rHBsAg)-loaded anionic NPs (protamine/HA of 1:4, w/w) induced the secretion of cytokines including TNFα, IL-1α, and IL-6 by macrophages more efficiently than the cationic NPs (protamine/HA of 4:1), whereas in mice, by either intramuscular or intranasal administration, the cationic NPs induced more robust immune responses than the anionic NPs did, as proved by the higher levels of the IgG

against the hepatitis B antigen in the cationic NP group, indicating that the protamine/HA NPs depending on physical features may be an effective VADS for delivering subunit HBV vaccines [74]. Kim et al. using LMW HA (215 kDa) synthesized HA-OVA conjugates, which proved able to facilitate DC maturation *in vitro* and, after topical application to penetrate into the dermis in murine skins, efficiently induced secretion of the anti-OVA IgG levels in serum as well as IgA levels in bronchioalveolar lavage, which could promptly respond to an OVA challenge after 8 weeks rendering a strong immune-recall humoral response, especially, under the condition of pretreatment of the skin using nonablative fractional laser beams to save Ag dose, strongly supporting of the adjuvant role that LMW HA can play for developing the painless topical VADS [75].

In summary, HA is a biodegradable and safe endogenous polymer, which can be used to engineer either inert NPs with high molecular weight HA or cell-targeting NPs with low molecular weight HA based on the fact its selective binding to several receptors, such as CD44 and TLR4, which may possibly trigger innate immune responses, allowing HA-based NPs to be conveniently employed to construct multifunctional VADS able to efficiently deliver various subunit vaccines.

3. Conclusions

At present, various polymeric NP-based VADSs have been designed for delivering as well as adjuvanting vaccines to elicit robust Ag-specific humoral and cellular immunity at both systemic and mucosal levels to provide extensive protection against infectious pathogens. In particular, many types of polymeric NPs can be tailored as a multiple functional VADS to render Ags lysosome escape after APC uptake, allowing vaccine epitopes not only to avoid being degraded into null pieces but also to selectively bind to MHC-I or -II for presentation to dictate immune responses toward a Th1 and/or Th2 pathway to set up immunity fitting medical aims. Encouragingly, a few of the polymeric NP VADS-based subunit vaccines have been approved, as mile stones, for clinical vaccination, typical products including the virosome-based hepatitis A vaccine (Epaxal®) and influenza vaccine (Inflexal V®), VLP-based HBV vaccine and malaria vaccine. Undoubtedly, as many of the uncertainties and problematic issues associated with polymeric NPs, such as safety of synthetic materials, scale-up production, and cost of products, are ultimately resolved, more and more polymeric NP VADS-based vaccines will be developed and licensed to enter markets.

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Conflict of interest

All the authors declared no conflict of interests.

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Chapter 4

Vaccines Developed for Cancer Immunotherapy

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Additional information is available at the end of the chapter

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Abstract

Vaccines have been successfully used for prophylaxis of infectious diseases for a long time and in the last decades have inspired researchers to make products with similar immunological mechanisms for cancer immunotherapy, which has been developed rapidly into clinical applications and has shown remarkable therapeutic efficacy, as exemplified by chimeric Ag receptor T cell (CAR-T cell) and immune checkpoint inhibitor-based therapies which can efficiently strengthen the body's immune system to fight against cancer, but they are also expensive. Therefore, encouraged by recent success of cancer immunotherapy, scientists are actively developing the low-cost tumor Ag-based vaccines, which, however, usually exhibit weak immunostimulating effects and, therefore, are often formulated with nanoparticulate carriers to form a vaccine adjuvant-delivery system (VADS), which can not only enhance the efficacy but also mitigate the off-target toxicity associated with conventional anticancer vaccines. These nanoparticulate carrier-based VADSs have demonstrated multiple functions, such as targetedly triggering Ag-presenting cells, reeducating tumor-associated macrophages (TAM) to function as tumor suppressor agent, and eliciting robust cytotoxic T lymphocytes (CTLs) to kill tumor cells. This chapter introduces multifunctional VADS that have been engineered with nanoparticulate carriers, including polymeric-, lipid-, metallic-, and cell-based nanoparticles, and used as an alternative to the existent tools for cancer immunotherapy.

Keywords: cancer immunotherapy, vaccine adjuvant-delivery system (VADS), immunoresponse, nanocarrier, cellular immunity, immune surveillance, danger-associated molecular pattern

1. Introduction

The huge success in vaccination against infectious diseases inspired researchers to explore the principles of immunotherapy for controlling tumor growth using host immune system,

which has been tried to be triggered with a variety of strategies, such as immune checkpoint inhibitor- and engineered T cell-based therapies, to elicit antitumor immunity in the body and has shown great potential in treatment and prevention of recurrence of cancer, as exampled by recent striking outcomes of cancer immunotherapy in clinical applications [1, 2]. From the overall view, current cancer immunotherapy is usually undertaken in two ways: establishment of systemic immunity through utilizing cytokines, vaccines, or adoptive cell transfer (ACT) and regulation of local immunosuppressive tumor microenvironment through utilizing small molecules and immune checkpoint inhibitors. Immune checkpoint therapy addresses regulatory pathways in preexisting Ag-specific T cells aiming at enhancing antitumor immune responses, whereas self-sustaining systemic anticancer immunity proceeds with anticancer immune response, which is dictated by both vaccines and TME [3]. However, immune checkpoint inhibitors are mostly aimed at augmenting the potency of preexisting tumor-specific T cells and as such benefit only a portion of patients [2], while the strategies based on the engineered T cells involve complex bioengineering processes with almost an unacceptable cost and, sometimes, off-target severe toxicities [4]. These situations compel researchers to develop other anticancer tools, including, especially, the tumor Ag-containing vaccines, which trigger the immune system to establish anticancer immunity through several crucial processes: release of Ags from tumor beds to be taken up by Ag-presenting cells (APCs) or delivery of Ags to APCs, APC activation for presentation of tumor Ags, priming and activation of T cells by activated APCs, migration and infiltration of effector T cells back to the tumor, and finally the recognition and killing of tumor cells by effector T cells, each of which, theoretically, can be targeted with various therapeutic approaches [5]. In particular, cancer vaccines that are designed for targeting early steps of Ag processing are potentially able to enhance both therapeutic and prophylactic efficacies against not only primary tumor but also inoperable metastasis or relapse and will therefore benefit a wide range of patients, especially the ones that lack sufficient levels of preexisting tumor-specific T cells and immune checkpoint-related molecules [6].

However, despite having in expectation tremendous therapeutic potential, cancer vaccines designed in conventional ways have been found elusive for successful treatment and eradication of tumors due to their weak immunogen insufficient to induce immune responses with conventional vaccination approaches. In addition, there are several other issues that may block the establishment of anticancer immunity, including degradation and rapid elimination of Ag, ineffective DCs uptake and Ag presentation, the suppression of T cell functions, and impairment of Ag presentation by the endoplasmic reticulum (ER) stress-driven lipid metabolism in DCs, thereby inhibiting protective T cell responses in cancer immunotherapy [7]. All of these highlight the need for developing new strategies to prepare cancer vaccines that can efficiently deliver tumor Ags and adjuvants to APCs and stimulate immune responses strong enough to kill tumor cells [8]. In this regard, NPs, such as liposomes, polymeric aggregates, and inorganic NPs, when used as a vaccine carrier have proven to be able to enhance the accumulation in draining lymph nodes (dLNs) of immunostimulants and adjuvant Ags, which thereby approach and stimulate a large number of Ag-presenting cells (APCs) enriched in dLNs to initiate cellular immunity required for fighting against cancer [6]. Moreover, several decades of intensive investigation on different types of NPs for targeting delivery

of traditional chemotherapeutics to solid tumors provide basis of repurposing these NPs to target the immune system and offer new opportunities to tune immunity and elicit strong antitumoral immune responses for overcoming the major challenge to the clinical translation of cancer vaccines [9].

Actually, multifunctional NPs show numerous advantages over conventional therapeutics for cancer immunotherapy: (1) NPs with finely tuned size and a defined surface features can achieve targeting delivery to lymphoid tissues, while most NPs composed of biomaterials bearing immune-stimulating properties may serve a dual role as a vaccine carrier and an adjuvant, thus forming a vaccine adjuvant-delivery system (VADS) to simplify the vaccine production [10–12]; (2) NPs carrying both tumor Ags and adjuvants can stably co-deliver vaccine components to APCs [13, 14]; (3) NPs can also be to display Ags and co-stimulatory ligands to serve as artificial APC and potentiate T cell immune responses [15]; (4) VADSs can be formulated to trigger immunogenic cell death or target immune checkpoint molecules leading to antitumoral immune responses and reverse of immune suppression [16]; and (5) VADS can also be loaded with therapeutics to improve antitumoral efficacy of adoptive T cell therapy [17].

This chapter elaborates new developments in areas of cancer immunotherapy, highlighting the potential of the VADSs engineered with various types of NPs for developing vaccines that are explored for cancer immunotherapy.

2. Cancer immunity and immunosuppression

Vertebrates are protected by the immune system from pathogens such as viruses, bacteria, fungi, and parasites through immune responses which can be classified into two categories, namely, innate and adaptive processes thus to establish, respectively, two types of immunity: the innate immunity providing rapid defense against pathogens and the more comprehensive adaptive immunity, which is set up requiring process of pathogens by professional APCs for presentation of immunogenic Ags to T and B cells to sponsoring cellular and humoral immune responses. Professional APCs, including B cells and macrophages, as well as dendritic cells (DCs) which are considered as the most efficient APC population, have proven to play a pivotal role at the interface of innate and adaptive immune responses [18, 19]. To initiate immune responses, DCs take up and then process endogenous or exogenous Ags in the context of major histocompatibility complex (MHC) class I or II, followed by presentation of the MHC-I or -II/Ag peptide complex as the activation "signal 1", respectively, to CD8+ and CD4+ T cells, which are activated requiring an additional "signal 2" induced by ligation of co-stimulatory markers CD80/86 on DCs with CD28 on T cells, as well as a T cell polarizing "signal 3" provided by cytokines, such as interleukins (ILs) and interferons (IFNs) secreted by DCs [6]. Although MHC-I is constitutively expressed by most mammalian cells, nonprofessional APCs can never present "signals 2 and 3" to alert the immune system after invasion by pathogens, highlighting the Ag processing and presentation by APCs as a critical first step in sponsoring adaptive immune responses. In particular, DCs rather than other APCs prove to be able to process exogenous pathogens to activate CD8+ T cells via a unique process called cross-presentation, of which, though the exact mechanisms are still unclear, the vacuolar and

cytosolic pathways have been identified to utilize endosomes and endoplasmic reticulum, respectively, to generate the MHC-I/Ag peptide complexes [20–22]. Notably, the endosome pathway in normal conditions is devoted to process the exogenous Ags to form the MHC-II/Ag peptide complexes for activation of CD4+ T cells, although the abnormal increase in endosomal pH or occurrence of endosome breakup made purposely by artificial strategies is thought to prevent the protease-mediated degradation of Ags in endosomes, thus promoting cross-presentation [12, 23], and additionally, certain DC subsets such as tissue-resident CD8+ and migratory CD103+ DCs in mice and CD141+/BDCA-3+ DCs in humans were reported to be more efficient at Ag cross-presentation than other DC subtypes [24, 25].

After activation and differentiation from CD8+ T cells in lymphoid tissues, the matured Ag-specific cytotoxic CD8+ T lymphocytes (CTLs) enter the systemic circulation and patrol peripheral tissues in search of target cells, which display a specific Ag epitope in the context of MHC-I matching the Ag-specific T cell receptors (TCRs) on CTLs, which once identification of the target cells will secrete perforin and granzymes to lyse them and within minutes move on to kill the next target [26]. By contrast, CD4+ T cells mainly play a helper role of regulation of immune responses as manifested by the observations that after activation by MHC-II/Ag peptide complex presented by DCs, naïve CD4+ T cells differentiate into four distinctive subtypes depending on the polarizing cytokines [27]. Type 1 helper T cells (Th1) induced by IL-12 secrete IL-2 and IFN-γ to promote CD8+ T cell responses; Th2 cells induced by IL-4 secrete IL-4 and IL-5 and are involved in humoral immune responses; regulatory T cells (Tregs) induced by IL-2 and TGF-β (transforming growth factor beta) secrete TGF-β and IL-10 to suppress immune responses; and Th17 cells induced by TGF-β, IL-6, and IL-21 secrete IL-17 and IL-22 to break immune tolerance and possibly leading to autoimmunity [27, 28]. In addition, it is reported that CD4+ helper T cells are utilizing the expressed CD40L for feeding back to DCs to further amplify immune activation and aid in establishment of memory CD8+ T cell responses [29, 30].

To prevent cancerous occurrence, the immune system constantly implements a process referred to as immunosurveillance whereby to inhibit oncogenesis by actively identifying and eliminating tumor cells, which however, have also devised mechanisms to evade immune responses, including downregulation of tumor Ags and promotion of immunosuppression [31, 32]. In established tumor microenvironment, it is generally immunosuppressive due to upregulation or production of inhibitory molecules, such as TGF-β1, CXCL12, VEGF, ARG1 (Arginase1), CCL18, iNOS (nitric oxide synthase), IL-10, IL-35, and galectin-1 by many types of cells, including cancer-associated fibroblasts, myeloid-derived suppressor cells, Tregs, and tumor-associated macrophages (TAMs), against T cells [33]. Also, activated T cells upregulate CTLA-4 (CTL-associated protein 4) which binds to co-stimulatory molecules on DCs with higher affinity than CD28, serves as a peripheral inhibitory signal to prevent over-reactivity of T cells, and dampens antitumor immune responses. Besides, tumor cells can also secrete cytokines such as IL-10 and TGF-β, which both directly inhibit the proliferation of CTLs and drive the differentiation of Tregs to provide an additional source of immunosuppressive cytokines, while subsets of tumor cells highly express programmed death-ligand 1 (PD-L1) for binding to programmed death-1 (PD-1) on T cells and inhibiting their effector functions [34]. Thus, tumor cells can promote immunosuppressive tumor microenvironment and shield themselves from CTLs by hijacking normal negative feedback loops designed to guard against

excessive activation of T cell responses, suggesting that development of vaccines for cancer immonotherapy is still confronting a huge challenge arising from cancer immunosuppression.

3. NP entrapping various Ags for delivering cancer vaccines

Tumor Ags include mutated cell surface components, such as polysaccharides, peptides, oncoproteins, and DNA and mRNA that encode those proteins, which as referred to subunit Ags, meanwhile tumor cell lysate and immunogenically dying tumor cells can also serve as the source of whole-cell Ags [6]. As key components utilized for formulating anticancer vaccines, subunit Ags have major advantages including defined chemical synthesis; ease of production; and for vaccine formulations, requiring, possibly, no Ag-processing by APCs and challenges including elicitation of humoral rather than cellular immune responses, poor delivery efficiency, and in vivo stability. Whole-cell Ags have major advantages including broad-epitope immune responses, potential for "personalized" therapy, full preservation of tumor Ags and challenges including production requiring tissue biopsy, difficulty in manufacturing, loss of antigenicity during production, presence of self-Ags, and immunosuppressive molecules such as PD-L1. Notably some viruses, such as Epstein–Barr virus (EBV), human papilloma virus (HPV), and hepatitis B and C viruses, have proven to contribute to certain cancer-related development, and therefore, their virally gene encoded surface proteins may also serve as the potential target Ags to constitute the vaccines for cancer immunotherapy [35, 36]. Among different types of tumor Ags, oncoproteins, which are encoded by oncogenes involved in the regulation or synthesis of proteins linked to tumor cell growth and may also be either mutated or overexpressed normal or embryonic proteins from fetal development, are intensively investigated for cancer vaccines since they have a big potential in induction of broad-epitope CD8+ and CD4+ T cell responses. Notably, compared to full-length protein-based Ags that require cellular uptake and processing for presentation to T cells, peptide epitopes can directly bind to MHC molecules and thus directly activate T cells and, moreover, are more endurable to damages during the preparation and storage of vaccine products, thus, in line with these advantages, leading to many ongoing clinical trials on peptide-based cancer vaccines [37, 38].

However, poor immunogenicity and limited therapeutic efficacy are still big challenges in developing protein, especially, peptide Ag-based subunit vaccines that are designed for cancer immunotherapy; for example, in the case of melanoma, the identified Ags include β-catenin, survivin, tyrosinase, gp100, MAGE, melan-A (MART1), and NY-ESO-1, some of which, such as gp100 and MAGE-A3 peptides, when tested in clinical trials just showed only moderate or null therapeutic efficacy [39]. Grooming through clinical trials on peptide-based cancer vaccines, it may be safely concluded that therapeutic efficacy of subunit vaccines against cancer remains suboptimal [2], due to at least partially the fact that many tumor Ags evaluated in clinical trials are self-Ags which can hardly trigger the autoreactive T cells leading to immunotolerance [6]. These disappointed outcomes highlight that the conventional subunit vaccines should actually be formulated with innovative modalities, which may be an alternative promising strategy to further improve cancer immunotherapy, as evidenced by positive results obtained from pre- and clinical investigations carried out more recently

on cancer vaccines that were combined with other elements, such as potent adjuvants and NP-based VADSs. For example, in a preclinical study, researchers observed that, in a syngeneic mouse model of oral cancer comprised of mouse tonsil-derived epithelial cells stably expressing HPV-16 E6 and E7 genes along with H-ras oncogene (mEER), intranasal HPV E6/E7 peptide vaccination or single checkpoint antibodies failed to elicit responses in most mice; however, 4-1BB agonist antibody along with either CD40 agonist antibody or CTLA-4 blockade eliminated the majority of established mEER tumors, and even produced a curative efficacy and a high safety profile against orally implanted mEER tumors [40]. For another example, in a phase II clinical trial, researchers performed immunotherapy with two peptide cancer vaccines in combination with intravesical bacillus Calmette-Guerin (BCG) for patients with non-muscle invasive bladder cancer (NMIBC) and demonstrated that this combinatory immunotherapy had good immunogenicity and safety and resulted in a 2 year RFS rate 74.0% in all patients, suggesting the cancer vaccines with a combinatory mode may provide benefit to patients for preventing recurrence of NMIBC [41].

These investigations showed that the conventional vaccines have limited capability to target delivery of tumor Ags and adjuvants to proper APC and intracellular compartments and may be renovated by the NP-based vaccine adjuvant-delivery systems (VADSs) which have already poised to address these challenges as described below.

4. NPs delivery of cancer vaccines

Cancer immunotherapy by vaccines depends on eliciting in patient the antitumor adaptive cellular immunity, which is, however, governed by potent Ag-presenting DCs able to activate CD8+ T cells and engender the Ag-specific CTLs. For this purpose, various immunotherapy strategies have been developed, including, in particular, using NP-based VADSs that are so elaborately designed as to promote APC cross-presentation of Ags and to deliver Ags and/or adjuvants targeting APCs, tissues, or organs such as dLNs, wherein APCs aggregate in large number ready for uptake of foreign substances, as can hardly be accomplished by soluble Ags or adjuvants alone [12].

4.1. NPs promoting Ag cross-presentation for delivering cancer vaccines

During an immune response, exogenous Ags are usually processed and presented via MHC-II by APCs to CD4+ T cells; however, tumor Ags engulfed by APCs require to be presented via MHC-I to induce production of Ag-specific CTLs, which are the main effector cells against tumor cells, thus precluding traditional methods from engineering cancer vaccines as they rely on soluble protein or peptide tumor Ags which often skew immune responses to CD4+ T cell responses while failing to induce robust CTL responses which are sufficient for cancer immunotherapy. Fortunately, it is disclosed that tumor Ags delivered by the elaborately designed NP-based multifunctional VADS able to promote lysosome escape, which is translocation of Ags from endosomes or phagosomes to cytosol avoiding Ag degradation within lysosomes, may regulate Ags to be reloaded to endoplasmic reticulum (ER)-attached MHC-I

for cross-presentation and favorably elicit CD8+ T cell responses [24]. As such, to engender Ag lysosome escape, great efforts have been focused on pH-sensitive delivery systems that can retain the loaded cargo under the physiological pH condition while triggering release of Ags and disruption of endocytic vacuoles at the acidic (below pH 6) endosomal microenvironment [42], as exemplified by a pH-sensitive liposomal VADS which is formulated with a dextran derivative and was shown to promote cytosolic delivery of Ags [43].

More recently, Wang and colleagues through fabricating two types of pH-sensitive multifunctional liposomes, the mannosylated lipid A-liposomes (MLLs) and the stealth lipid A-liposomes (SLLs) both loaded with Ags and NH_4HCO_3, into microneedles prepared the proSLL/MLL-constituted microneedle array (proSMMA), which dissolved rapidly recovering the initial MLLs and SLLs upon rehydration [12]. Mice vaccinated with proSMMAs by vaginal mucosa patching elicited robust Ag-specific humoral as well as cellular immunity at both systemic and mucosal levels, especially, in the reproductive and intestinal ducts. Further exploration revealed that the Ags delivered by either liposomes were cross-presented for MHC-I displaying by APCs thanks to lysosome escape and reactive oxygen species stimulation, both of which occurred when lysosomal acidifying the liposome-released NH_4HCO_3 into CO_2 and NH_4^+/NH_3 to rupture lysosomes by gas expansion and to cause ROS production by excessive ammonia induction, resulting in a mixed Th1/Th2 type response which was also promoted by liposomal lipid A via activation of TLR4, indicating the proSMMAs a multifunctional VADS capable of engendering Ag lysosome escape to elicit robust humoral and cellular immunity against Ags and a promising platform for making both cancer and infection vaccines.

In addition, an alternative approach for evading lysosome degradation of Ag includes multifunctional VADS constituting of the oxidation-sensitive polymersomes that can respond to the oxidative environment of endosomes and deliver Ags and adjuvants to cellular cytosol for induction of cellular immune responses [44]. Notably, liposomes modified with a cell-penetrating peptide octaarginine were also reported to be able to promote cross-presentation Ags and elicit production of anticancer CTLs, because the membrane-penetrating liposome enhanced proteolysis of the exogenous Ags by proteasomes and amino peptidases facilitating promoting the C-terminal trimming of antigen peptide and the production of mature MHC-I peptides [45]. Also, gold nanoparticles displaying tumor Ags were reported to enable efficient antigen delivery to dendritic cells and then activate the cells to facilitate cross-presentation, inducing Ag-specific CTL responses for effective cancer immunotherapy [46].

4.2. NPs targeting DC for delivering cancer vaccines

Recently, the approach based on amphiphilic polymer-Ag peptide conjugates through the conjugation of azide-functionalized Ag peptides to an alkyne-functionalized core via azide-alkyne click chemistry has been employed for making nanovaccines against cancer. For example, by conjugation of the melanoma Ag peptide TRP2 and azido PEG mannose to the alkyne polymer, an anti-melanoma nanovaccine with the size of 10–30 nm was formed via self-assembly and was efficiently taken up by DCs [47]. In spite of poor immunogenicity, when given to model mice with B16-F10 melanoma tumors together with the adjuvant CpG, the adjuvanted TRP2-nanovaccines effectively suppressed the tumor growth and significantly

improved the survival of mice compared to the untreated group. Moon's group engineered synthetic high density lipoprotein (sHDL) nanodiscs consisting of phospholipids, apolipoprotein A1 (Apo A1)-mimetic peptides and cholesterol-conjugated CpG (sHDL-Ag/CpG) with average diameter of 10 ± 0.5 nm, which were used as a multifunctional VADS able to target lymphoid organs, resulting in sustained Ag-presenting on DCs [48]. Moreover, the sHDL-CpG-based VADS loaded with multiple Ags (MHC-I-restricted M27, MHC-II-restricted M30, and TRP2) in combination with anti-PD1/anti-CTLA4 antibodies successfully rejected B16-F10 tumor from tumor-bearing mice.

Though targeting delivery with NPs is able to improve efficacy of cancer vaccines, tumor-induced DC dysfunction arising from hyperactivity of signal transducer and activator of transcription 3 (STAT3) [49], which leads to less maturation in DCs with low responsiveness to pattern recognition receptor agonist (PRRa) stimulation [50], engenders another major huddle to developing effective vaccines for cancer immunotherapy. The NPs-based VADS was trialed in overcoming tumor-induced DCs dysfunction by Ma and colleagues through using poly(ethylene glycol)-b-poly(L-lysine)-b-poly(L-leucine) (PEG-PLL-PLLeu) to form 120 nm-sized polypeptide micelles for encapsulation of polyI:C, STAT3 siRNA, and OVA as a nanovaccine (PMP/OVA/siRNA), which proved able to decrease STAT3 expression and increase CD86 and CD40 expression as well as IL-12 production [51]. Moreover, PMP/OVA/siRNA nanovaccine could effectively increase mature DCs and decrease immunosuppressive cells in tumor draining lymph node, leading to antitumor immune response and prolonged survival, implying that novel VADSs designed for co-delivery of immunopotentiator and immunosuppressive gene silencer may be one of potent strategies to improve antitumor immunity by modulating tumor-induced DCs in tumor microenvironment.

4.3. NPs targeting the lymph node for delivering cancer vaccines

Pattern recognition receptors (PRRs) are germline-encoded host sensors expressed mainly by cells of the innate immune system, such as MPs, (MPs) macrophages, monocytes, neutrophils, and epithelial cells, capable of detecting two classes of molecules: pathogen-associated molecular patterns (PAMPs), which are associated with microbial pathogens, and damage-associated molecular patterns (DAMPs), which are associated with components of host's cells that are released during cell damage or death [52]. PRRs play a crucial role in the proper function of the innate immune system evolved before other parts of the immune system, particularly before adaptive immunity, and mediate the initiation of Ag-specific adaptive immune response and release of inflammatory cytokines when they are activated by PRRas (PRR agonists), which are the microbe-specific molecules, including bacterial carbohydrates such as lipopolysaccharide (LPS) and mannose, bacterial peptides such as flagellin and microtubule elongation factors, peptidoglycans and lipoteichoic acids, nucleic acids such as bacterial or viral DNA and RNA, fungal glucans, and also chitin and thus are often used as vaccine adjuvants.

Since a fraction of PRRas such as LPS and unmethylated CpG ODN are soluble, prevention of rapid diffusion of free PRRas into the systemic blood circulation is indispensable

for efficient targeting to professional APCs, which may be well obtained through formulating into the NP-based VADSs. This has been accomplished by dextran-CpG-OVA conjugate that enhanced not only the CD8+ T cell responses but also improved the antitumor immunotherapy through whole tumor cell vaccine. Recently, Liu and coworkers using reductive amination method conjugated oxidized dextran to amine-modified CpG ODN and demonstrated that the dextran-CpG conjugate with a hydrodynamic diameter of 6.5 nm was accumulated dLNs and was efficiently taken up by mouse DCs [53]. With the combination of OVA as a model Ag, dextran-CpG conjugate elicited production of Ag-specific CD8+ T cells for effective therapeutic benefits and in subcutaneously immunized mice resulted in significant reduction of tumor growth and increased survival of mice.

To induce a potent MHC-I-restricted CTL response which is an essential component of the successful cancer immunotherapy treatment, Huang's group formulated the mannosylated lipid-calcium-phosphate (MLCP) NPs as a new class of intracellular delivery systems for cytosol delivery into DCs of an exogenous Ag, p-Trp2 (the melanoma Ag Trp2 peptide derivative bearing two phosphor-serine residues) [54]. Compared with free Trp2 peptide/CpG ODN, MLCP NPs encapsulation enhanced and prolonged the cargo deposit into the lymph nodes (LNs) and also resulted in superior inhibition of tumor growth in both B16F10 subcutaneous and lung metastasis mouse models owing to induced IFN-γ production and a Trp2-specific CTL immune response. Thus, encapsulation of phospho-peptide Ags into LCP may be a promising strategy for enhancing the immunogenicity of poorly immunogenic self-Ags for cancer therapy.

Recently, a nanovaccine, called AlbiVax that is assembled in vivo from endogenous albumin nanocarriers and exogenous molecular vaccines, which are chemically defined and relatively well suited to large-scale production including quality control and safety evaluation, has been developed based on the albumin properties which are well known of not only being efficiently internalized by APCs via endocytosis to facilitate intracellular vaccine delivery for optimal Ag processing and presentation but also binding to a clinically practiced Evans blue (EB) [55]. AlbiVax was synthesized by conjugating thiol-modified vaccines and adjuvants, such as the 3′-end thiol-modified CpG and Ags (CSIINFEKL, Trp2, and Adpgk) modified with N-terminal cysteine, with maleimide-functionalized EB derivative which can tightly bind to human serum albumin. Further investigation revealed that, compared to benchmark incomplete Freund's adjuvant (IFA), AlbiVax had a much high efficiency in co-delivery of CpG and Ags to LNs and in eliciting peripheral Ag-specific CD8+ CTLs with immune memory and specifically inhibited progression of established primary or metastatic EG7.OVA, B16F10, and MC38 tumors; but only in combination with anti-PD-1 and/or Abraxane did AlbiVax eradicate most MC38 tumors. These outcomes indicate that as a novel type of VADS, the in vivo self-assembled molecular nanovaccines can not only enhance vaccine bioavailability in LNs but also bypass the complications, such as inefficient delivery, sequestering Ag determinant-specific T cells in the depots, and exhausting and depleting T cells, thereby preventing T cells from infiltrating tumors and difficulty in large-scale production, which are often associated with conventional synthetic vaccines [56].

4.4. NPs in combination for delivering cancer vaccines

The VADs based on various NPs that are engineered with different adjuvants such as PRRas and tumor Ags in combination for delivery of vaccines to the same immune cells have a great potential in provoking immune responses, generating increased duration and speed of immune response, regulating Ag-antibody response, and amplifying immunogenicity of weak Ags [12]. For example, it is reported that the application of poly(γ-glutamic acid)-based NPs for the delivery of model Ag (OVA) and toll-like receptor 3 (TLR3) agonist poly (I:C) (polyinosinic-polycytidylic acid) in targeting the LNs significantly enhanced the antitumor immunity against EG7-OVA (EL-4 thymoma cells transfected with chicken albumin cDNA) in tumor-bearing mice [57]. Recently, Molino et al. designed a biomimetic approach for eliciting antitumor responses through engineering the viral-mimicking protein NP vaccine, which is pyruvate dehydrogenase E2 protein NP (of 50 nm) conjugated to gp100 epitope (melanoma-associated Ag) and CpG [58]. The CpG-gp-E2 NPs remarkably increased the proliferation of Ag-specific CD8+ T cells and production of IFN-γ and dramatically enhanced the population of CD8+ T cells in dLNs, resulting in the delayed onset of tumor growth in mice as well as elevated mouse survival, compared to control PBS-treated animals.

It should be pointed out that delivery of vaccine Ags and adjuvants to target tissues or cells by a VADS is also, to a great extent, dictated by NP properties, such as particle size and surface charge, which may be appropriately engineered for improving their delivery efficiency [59]. For example, the NP-based vaccines could be either delivered actively to the lymph nodes by DCs in target tissues or transported by the interstitial flow into the lymphatics, depending mainly on NP size and surface properties such as PEGylation and charge due to the upper limit of pore size of the lymphatic capillaries and cell uptake of NPs relevant to surface properties of both [12, 60]. Wang's group engineered two types of multifunctional liposomes, the mannosylated lipid A-liposomes (MLLs) with a size of 200 nm and the stealth lipid A-liposomes (SLLs) of 50 nm, both of which were loaded with a model Ag and NH_4HCO_3 and fabricated into microneedles, forming the proSLL/MLL-constituted microneedle array (proSMMA) as a multifunctional VADS [12]. Mice vaccinated with proSMMAs by vaginal mucosa patching administration established robust Ag-specific humoral and cellular immunity at both systemic and mucosal levels, especially, in the reproductive and intestinal ducts, under the revealed mechanism that the MLLs reconstituted from the administered microneedles were mostly taken up by vaginal mucosa resident DCs, whereas the recovered SLLs trafficked directly to dLNs wherein they are to be picked up by macrophages, proving the size of NPs as an important parameter in controlling the in vivo fate of the delivered vaccines.

5. NPs for delivering DNA and mRNA vaccines

Using DNA and mRNA for intracellular production of oncogenic proteins or peptides as tumor Ags becomes an attractive strategy for developing cancer vaccines thanks to the advances in biotechnology which allows gene encoding proteins of interest to be easily manufactured in batch and be further modified with nucleic acid sequences that encode for proteins with immunostimulatory functions, for example, flagellin and a toll-like receptor

5 agonist (TLR5a). Unfortunately, previous clinical trials on DNA cancer vaccines, majority of which were administered as naked DNA via the intramuscular route, showed generally poor response rates, despite employment of viral vectors and electroporation able to improve the transfection of DNA vaccines, both of which cause safety and compliance concerns [61, 62]. Alternatively, NPs engineered as a VADS for intracellular delivery of DNA and mRNA provide a promising strategy for developing nucleotide-based cancer vaccines and possess several advantages [6]: (1) synthetic material-constituted NPs are safer than viral vectors, (2) NPs can stabilize and protect gene therapeutics from nuclease-mediated degradation [63], (3) DNA- and RNA-loaded NPs can be administered by injection-free tools, such as microneedles for non-parenteral delivery [64], and (4) nanocarriers can be easily modified with targeting moieties, for example, mannose, to achieve DC-targeted delivery and transfection [14, 65].

It is reported that cationic liposomes and lipid nanoparticles containing mRNA coding for the tumor-associated Ags gp100 and TRP2 could induce a strong CD8+ T cell activation after a single immunization and treatment of B16F10 melanoma tumors with the mRNA-carried cationic liposomes resulted in tumor shrinkage and extended the overall survival of the treated mice, all of which could be further increased by the combinatory incorporation of the adjuvant LPS, showing the cationic liposomes a promising vector for mRNA vaccine delivery that is capable of inducing a strong cytotoxic T cell response for cancer immunotherapy [66]. Nevertheless, nucleotide-based vaccines, including DNA and mRNA vaccines with their intracellular Ag synthesis, have been shown to be potent activators of a cytotoxic T cell response which is an important prerequisite for successful immunotherapy against many viral diseases and tumors [67], though intracellular delivery of mRNA vaccines to the cytosol of APCs is still not sufficiently well understood and remains somewhat a challenge to clinical translation for cancer immunotherapy [68].

6. NPs for overcoming immunosuppression

With great advances in immunology and oncology, several mechanisms, involving multiple immune components, have been identified to contribute to tumor immune escape, as summarized by Chabanon and coauthors as these including [69]: (1) reduction of MHC-I molecule expression in malignant cells, resulting in decreased antigen presentation and consequently reduced detection by CTLs; (2) induction of immune cell apoptosis by cancer cells through the expression of death signals; (3) release of a variety of immune-modulatory molecules such as IL6 and IL10 by tumor cells in the microenvironment to induce immunosuppressive Tregs while inhibiting the activity of CTLs; (4) secretion of TGF-β, COX-2 (cyclooxygenase-2), and PGE2 (prostaglandin E2) by tumor cells inhibiting DC differentiation and maturation while favoring the establishment of an immunosuppressive tumor microenvironment; (5) upregulated expression of immune checkpoint ligands to activate immune checkpoint receptors providing co-inhibitory signals to CD4+ and CD8+ T cells preventing them from building a specific antitumor immune response.

Among these elements involved in cancer resistance, immune checkpoints are regulators of the immune system to provide pathways crucial for self-tolerance and thus play an important

role both in the prevention of autoimmunity refraining the immune system from attacking cells indiscriminately under normal physiological conditions and in the regulation of immune reaction to avoid tissue damages during the pathogenic infection. Under normal conditions, immune checkpoints function via the interaction between a receptor expressed on T cells and its ligand located at the surface of APCs to generate a co-stimulatory signal, which triggers either the activation or inhibition of T cells. Presently, two major checkpoints have been clearly identified to regulate T cell activation: (i) the CD28/CTLA-4 axis, which activates T cells upon engagement of CD28 with CD80 and CD86, and conversely inhibits T cells when CTLA-4 is engaged and (ii) the PD-1 axis, which provides a strong inhibitory signal following binding of PD-L1 or PD-L2 to the PD-1 receptor [70]. Contrary to CTLA-4, PD-1 is thought to act predominantly in the tumor microenvironment, where PD-L1 is overexpressed by multiple cell types, including dendritic cells, M2 macrophages, and tumor-associated fibroblasts [71]. Thus, immune checkpoints and pathways, unfortunately, are also utilized by cancer cells as a key mechanisms to realize immune escape through upregulated expression of immune checkpoint ligands and as such deregulation of immune checkpoint signaling to suppress T cell activity in tumor microenvironment, a phenomenon that has been observed in multiple malignancies. Moreover, immune checkpoint molecules have been shown to promote the epithelial-mesenchymal transition of tumor cells and the acquisition of tumor-initiating potential and resistance to apoptosis and antitumor drugs, as well as the propensity to disseminate and metastasize, and thus have been increasingly considered as a crucial target for cancer immunotherapy given their potential for use in multiple types of cancers. Notably, as opposed to other immune-based approaches developed to fight cancers, immune checkpoint blockers (ICBs) have displayed significant therapeutic successes in many solid tumors and hematologic malignancies, as exampled by several anti-PD-(L)1-based drugs, such as the anti-CTLA-4 ipilimumab (by Bristol-Myers Squibb), the anti-PD-1 pembrolizumab (by Merck), and the anti-PD-L1 atezolizumab (by Genentech/Roche), durvalumab (by AstraZeneca/MedImmune), and avelumab (by Pfizer), all of which have already been approved for cancer immunotherapy [69].

However, with the current antibody-based immune checkpoint therapy, the nonspecific accumulation of antibody in the normal organs and tissues may ignite overreactive immune responses, which may even damage the body and cause severe side effects [72]; suggesting targeting delivery may provide beneficial effects even in the antibody-based immunotherapy. Recent studies have shown that a diverse set of NPs that have been engineered to improve delivery efficiency of immune checkpoint modulators which possess the potency in enhancement of the anticancer efficacy of the immune checkpoint blockade-based immunotherapy. Using a common procedure of water-in-oil-in-water emulsion, Wang's group formulated cationic NPs loaded with CTLA4 siRNA (siCTLA4) which was to modulate immune suppression mechanism [73]. The siCTLA4-NPs delivered siRNA into the T cells reducing mRNA and protein levels of CTLA4 upon the T cell activation in vitro and, when systemically given to mice, significantly increased the number of both CD4+ T cells and CD8+ T cells, whereas the number of CD4+ FOXP3+ regulatory T cells were decreased, resulting in the inhibited tumor growth and prolonged survival rate of B16 mouse melanoma model. PD-L1 is expressed on a variety of tumor cells, such as melanoma, NSCLC, ovarian cancer, head and neck cancer, B cell lymphoma, and thymic cancer and therefore is another attractive target for immune checkpoint modulation, which can be realized using tumor-targeted delivery system loaded

with relevant functional molecules. Yang et al. engineered folic acid-modified NPs with polyethyleneimine (PEI) derivatives and demonstrated that PD-L1 siRNA-loaded PEI NPs efficiently inhibited PD-L1 expression on SKOV-3-Luc tumor cells, resulting in sensitizing tumor cells to T cell killing in vitro [74]. Considering cancer recurrence after surgical resection remains still a significant challenge and platelets can accumulate in wound sites and interact with circulating tumor cells (CTCs) triggering inflammation and repair processes in the remaining tumor microenvironment, Gu's group engineered the anti-PD-L1 antibody-conjugated platelets (P-aPDL1) which were employed to reduce postsurgical tumor recurrence and metastasis [75]. In mouse models bearing partially removed primary melanomas (B16-F10) or 4T1 (triple-negative breast carcinomas), B16-F10 effectively released anti-PD-L1 upon platelet activation by platelet-derived microparticles and remarkably prolonged overall mouse survival after surgery by reducing the risk of cancer regrowth and metastatic spread, suggesting engineered platelets an efficient VADS which can facilitate the delivery of the immunotherapeutic anti-PD-L1 to the surgical bed and target CTCs in the bloodstream to improve the objective response rate.

Summarily, the VADSs based on various NPs that are engineered to bear therapeutic functions are promising in targeted delivery of the immunomodulatory agents to offset the immunosuppressive effects generated in tumor microenvironment and to rehabilitate the defensive immunity, maximizing the efficacy of cancer immunotherapy while minimizing side effects.

7. Conclusions

In recent years various types of NPs have been designed as a VADS for delivery of vaccines that are aimed for cancer immunotherapies and have shown great promise in curing refractory tumors which can never be obtained by conventional clinical measures, such as chemotherapeutics, surgery, and radiation. The NP-based cancer VADS possesses numerous advantages, including high safety profile and thus good compliance, high stability, diverse administration routes, and ease in modification with functional molecules as well as large-scale production, and bears also disadvantages including mainly relatively weak immunostimulatory capacity and low intracellular especially intranuclear delivery efficiency, which may be hopefully overcome by elaborate design with adjuvants such as PRRas and multifunctional molecules. Nevertheless, the NP-based cancer VADS proves able to successfully elicit antitumor immunity both in vitro and in vivo through, in particular, targeting APCs and draining lymph nodes, engendering lysosome escape, and modulating immunosuppression and represents new directions in developing efficient tools for cancer immunotherapy.

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Conflict of interest

All the authors declared no conflict of interests.

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Chapter 5

Implementation of the Expanded Program on Immunization (EPI): Understanding the Enablers and Barriers in a Health System

Babar Tasneem Shaikh

Additional information is available at the end of the chapter

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Abstract

Much has been written about the issues, challenges and constraints in the implementation of immunization program. However, there is a need to better understand the health system barriers as well as enablers that influence the progress. This chapter aims at bridging the information gaps about system-level factors that currently are impeding the optimal delivery and uptake of immunization services to the children through the Expanded Program on Immunization (EPI). This chapter draws its thematic content from a critical review of the EPI-related international and national reports. In addition to this, this chapter consulted government reports, surveys, and publications on health system. Themes generated from the literature review included financing, governance, service delivery, human resources, information system, and supplies and vaccines. Findings suggest that certain areas in the larger health system are to be improved for a more coordinated implementation of EPI. It is imperative to understand community's behaviors and perceptions as well as demand side issues in order to achieve the desired results. For a better immunization coverage, EPI operations and performance must be improved. Implementation research could definitely help in developing an even finer understanding of the system-wide factors influencing the efficiency of the program.

Keywords: immunization, child health, health system, implementation research

1. Introduction

Despite being an established cost effective public health strategy for improving child survival, each year millions of children in low- and middle-income countries (LMICs) do not receive

the full series of vaccines on their national routine immunization schedule [1, 2]. In Pakistan, over 50% of deaths in post-neonatal children are attributable to pneumonia, diarrhea, or meningitis, which can be prevented through vaccination [3]. The Government of Pakistan initiated the Expanded Program on Immunization (EPI) in 1978, and gradually introduced all requisite antigens, with the recent addition of Rota virus [4]. WHO recommends immunization coverage of 90% at the national level and at least 80% for every district [5]. Pakistan's immunization indicators have improved since the program's inception; however, recent data from 2012 to 2013 recorded merely 54% full immunization coverage for children age 12–23 months (**Figure 1**) [6].

Vaccine-specific coverage starting from BCG coverage at 85% falls to 61% for measles (**Figure 1**). In addition, there is a large drop out seen from the first two doses of polio (90.2%) and DPT (76.8%) to third doses of the same vaccines (82% and 62.5% respectively). Vaccine coverage drops with birth order; first child coverage is 64% while only 39% of children born in order 6 or more are fully covered. There are significant regional variations with the Islamabad Capital Territory having the highest percentage (74%), followed by the provinces of Punjab (66%) and Khyber Pakhtunkhwa (53%); whereas immunization coverage is lowest in Sindh province (29%) and Baluchistan province (16%). There are obvious differences in immunization coverage between children of women with no education (40%) and children of literate mothers (74%). Children from households in the highest wealth quintile are much more likely to be fully immunized (75%) as compared to those in the lowest quintile (23%) [6]. In Punjab, the situation seems to be deteriorating (**Figure 2**) with the percentage of fully immunized children age 12–23 months dropping to be 56% in 2014 [7], whereas Sindh showed improvement with full immunization coverage increasing to 35% in 2014 [8].

Another national survey from 2014 to 2015 captured a significant gap in the percentage of fully immunized children between rural (56%) and urban (70%) areas. The provincial differences demonstrate similar disparity. The data for urban/rural differences by province were in

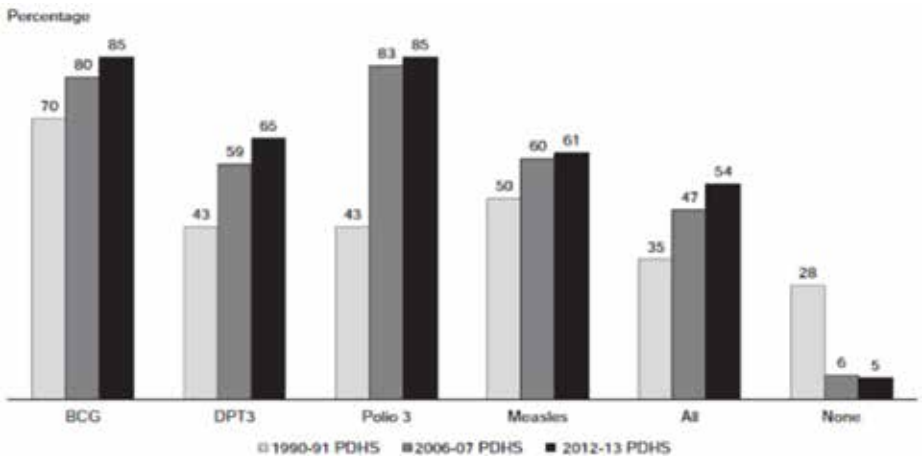

Figure 1. Trends in immunization coverage among children age 12–23 months. Pakistan Demographic & Health Survey 2012–2013.

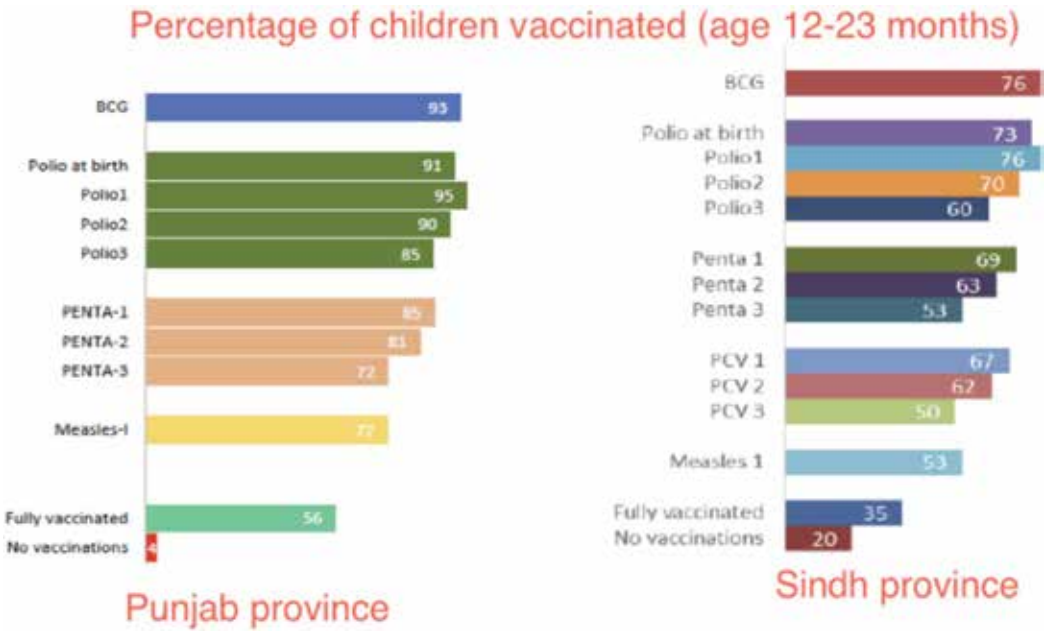

Figure 2. Vaccination coverage in 2014 for children age 12–23 months in Punjab and Sindh provinces. Multiple Indicator cluster survey 2014.

Sindh (62/33%), Baluchistan (48/20%), Khyber Pakhtunkhwa (74/54%), and Punjab (75/65%). Punjab had the highest immunization rate (70%) followed by Khyber Pakhtunkhwa (58%) and Sindh (45%). Baluchistan, which is the most deprived area, had the lowest coverage with only 27% of children fully immunized [9].

Given this state of affairs, it is evident that there is a need to take stock, particularly to understand the health system wide enablers as well as the barriers that could influence progress. And thereon develop strategies to either overcome or capitalize on these factors to optimize performance of the EPI program.

This chapter aims at bridging the information gaps about system-level barriers that currently are impeding the optimal delivery of immunization services to the children of Pakistan. We employed the basic tenets of WHO's health systems strengthening framework i.e. governance, financing, service delivery, human resource, information systems, and essential drugs, supplies and technologies [10], and the Sallis' socio-ecological model which helps in studying the community's perceptions and behaviors [11]. Hence, this study explored various pillars of the immunization program in Pakistan from both the service delivery and the demand side perspective. We conducted a detailed literature review to document what has been published already about this topic, identified barriers and levers of EPI implementation, and then developed a set of recommendations. Using MeSH terms and key words (Immunization; Child health; Health system; Pakistan), relevant peer reviewed articles were accessed using PubMed and Google Scholar. Other reports and documents were accessed from the websites of EPI Pakistan and UN agencies. Salient areas emerging from the literature review were cataloged under the building blocks of the health system.

2. Factors influencing EPI implementation

The information gleaned from the peer-reviewed articles, government reports, EPI documents, WHO/UNICEF/GAVI reports and some gray literature, unravels a stagnant or declining immunization status. At the same time, this analysis also shows a multifactorial picture responsible for the current state of affairs of EPI in Pakistan.

2.1. Program financing

Development partners have always generously supported the maternal, child and newborn health programs in Pakistan [12]. Although the immunization program mainly depends upon domestic development funds, resources from donors (WHO, UNICEF, GAVI, etc.) have been instrumental too. Pakistan is the biggest recipient of GAVI at present, categorized as a Tier 1 priority country. GAVI financial support to the Pakistan government has been channeled through partner organizations, predominately, WHO and UNICEF [13]. The Government of Pakistan's own share represents approximately 20% of the total EPI allocations [6]. The Japan International Cooperation Agency (JICA) and the World Bank also support the program. Moreover, the donors have supported in-service trainings for EPI managers. However, delayed release of funds and inefficiencies in expenditure have been noted as some key issues in the past. Of particular note is the lack of appropriation for transportation and fuel costs. Shortage of funds for repair and maintenance of cold chain equipment and vehicles could jeopardize vaccine efficacy [13].

2.2. Program governance

Pakistan went through devolution of its services related public sectors including health sector with the 18th amendment in its constitution effective from June 28, 2011. The Federal Ministry of Health (MoH) was dissolved and the overall responsibility for health services policy direction and planning was devolved to the provinces [14]. Inefficiencies and stalled health system's performance was observed at nearly all of the operational levels for some time after the devolution of 2011 [15]. A lack of clarity in roles and responsibilities of federal and provincial tiers of the government resulted in a vacuum in governance, and weak stewardship at decision-making levels The National Health Vision 2016–2025 later outlined more clearly the roles and responsibilities of the federal and provincial government vis-à-vis health programs and interventions [16]. There is a proposal that going forward, each district must have its own EPI implementation plan, which should consider and address the gaps identified by the situational assessment [13]. Polio in Pakistan has generated much analysis and discussion at the global and national levels. In late 2014, due to a rise in polio cases, an Emergency Operations Centre was established, and was mandated to ensure a synergy between the Polio Eradication Initiative and EPI, as well as with other sectors. Nevertheless, this convergence or synergy is still to be seen as fully operational [17]. The role of the private sector as a key stakeholder has also been documented with regard to governance of EPI [18], which could work hand in hand with the public sector in order to achieve the desired targets of immunization; but its potential still remains untapped.

2.3. Human resource

The lack of a comprehensive human resource (HR) strategy has been discussed time and again in the context of EPI in Pakistan. There is no regular and formal training program for the management cadres, and learning is mostly self-directed and on the job. Managers often lack the practical knowledge for leading program operations proficiently [19]. EPI workers' fatigue due to frequent polio campaigns have reduced their time dedicated to routine EPI vaccination initiatives [13]. In-service training for routine immunization staff is not held on the basis of any planning and programming, rather it is conducted whenever the donor funding is available. Competency of the staff, outreach capacity, service structure, attitudes toward clients, political interference in transfers and postings, and lack of accountability are all notable HR related issues of EPI [20, 21]. On the other hand, there are workers in the Polio program who are willing to perform their duties while putting their lives at risk, and facing extremist sections of the society, amidst a grim law and order situation [22]. Introducing incentive structures among managers and health workers of EPI or contracting with non-governmental organizations (NGOs) can potentially improve the HR performance [23].

2.4. Service delivery

Immunization services provided through outreach are costly and face logistic issues. The outreach strategy of EPI lacks details in micro-plans; has weak monitoring and supervision; and deficient human, operational, and other resources [13]. In many rural areas, routine immunization literally comes to naught during National Immunization Days, when all vaccinators are entrusted with the additional responsibility of covering 150–200 children per day, door-marking, record keeping in tally-sheets, and locating and marking missing children [24]. Coverage of vaccination services requires a rational re-deployment of vaccinators, and task-shifting to community-based service providers e.g. lady health workers (LHWs) and community midwives for covering their catchment areas. Vaccinators would thus be able to focus on areas not covered by any workers [25]. Involvement of the private sector and NGO outlets is also one of the solutions, but at present there is no policy in EPI on formal engagement with the private sector [13].

2.5. Supplies and vaccines

Interrupted supply of vaccines has been reported from time to time. Delays in forecasting, procurement, storage, and distribution to the provinces, districts and to the "last mile" (i.e. the hardest to reach segments of the population) have suffered in the past because of unduly tedious procedures [13]. Inadequate maintenance of cold chain is another issue reported in the literature. Power outages are frequent and there is no electricity back up at many places. EPI has state of the art cold chain for vaccine storage and transport; however, its maintenance has been a long-standing issue, particularly in rural remote areas where program monitoring is also weak [26]. Alternative solutions such as solar energy ought to be tried as a backup for power outages.

2.6. Information systems

Unreliable reporting, poor monitoring and supervision systems, and limited use of local data for decision-making are other impediments in the performance of EPI. Data collection is paper-based at the facility level, and then from district upwards, it becomes electronic. Therefore, establishing its credibility has been a challenge. Moreover, for quite some time, the EPI data was not reflected in the district health information system (DHIS) [27]. Inaccurate immunization records lead to the loss of billions of rupees every year [15]. There is a dearth of health systems research to better understand the dynamics between EPI and the beneficiary population [28].

2.7. Community perceptions and behaviors

Low community awareness and misbeliefs that vaccines cause disease, and the doubts about vaccine safety and effectiveness have been reported as important factors, impeding the uptake of immunization, especially in case of polio [29]. Therefore, educating the masses and population segments with low literacy levels, especially the women, is a must for improving the utilization of immunization services [30]. Gender differential in immunization coverage needs innovative gender mainstreaming strategies at the community level such as employing more female vaccinators and community volunteers for outreach to women [31]. Community activists can also encourage people to seek immunization services, and can increase demand through educating various community segments [32]. Communication between immunization workers and the parents of children has been flawed, and a positive engagement has helped with overcoming the resistance to vaccinations [33]. On the other hand, service providers in clinics do not emphasize the importance of immunization [34]. Religious beliefs and lack of knowledge about the benefits of the vaccines still dictate many pockets of this highly diverse and populated country [35]. Targeted community awareness programs, a robust surveillance network, and engagement with the dominant religious entities can help to root out the issue [36, 37]. Better understanding of the religion and soliciting local support for vaccination campaigns may assist in negotiating access in the areas where refusal is an issue [38].

Issues	Demand/supply side barriers
1. Low awareness level among caregivers and healthcare providers regarding vaccine-preventable diseases and their risks	Demand
2. Concerns of caregivers about safety of Oral Polio Vaccine	Demand
3. Belief in and use of local remedies for prevention and treatment	Demand and Supply
4. Low knowledge and awareness of health care workers regarding VPDs and their prevention	Supply
5. Distance, time and cost of travel to health facility and long waiting time there	Demand and Supply
6. Unavailability of vaccines and vaccinators and dissatisfaction with quality of service	Demand and Supply
7. Missing vaccination card in the home	Demand

Table 1. Demand and supply side barriers in effective implementation of EPI.

Demand side issues and community misperceptions are quite high. Ample funds are allocated for social mobilization, yet meager amounts are spent on communication, and to create community awareness of routine immunization [13]. Moreover, a shift of resources from mass media (TV and radio) to community-level, dialogic communication is proposed, given clear evidence that caregivers rely on healthcare providers, family and friends for information about immunization [16]. The demand and supply barriers of EPI have been well summarized (**Table 1**) in an important study undertaken by UNICEF [39].

3. Discussion and recommendations

There are several factors which we can bank upon for improving the EPI immunization program in Pakistan: provincial autonomy as called for in the 18th constitutional amendment, re-enactment of a national ministry of health for coordination, the infrastructure needed for the polio program and the renewed focus of the government and the development partners on routine immunization. No program, however, can improve without looking at it insightfully and searching for the underlying factors that may be the reason for its sub-optimal performance. This monograph has unraveled some important areas that need further exploration. These areas along with key recommendations are summarized here for future research and to broaden the evidence base for the immunization program in Pakistan and elsewhere.

1. *Financing and resource allocation: The budgetary allocations, spending and reporting has to be made more efficient.* Switching over to a midterm budgetary framework mode could be a good option for EPI. This mode of financing will be performance-based and target-oriented. Funds must be earmarked for the maintenance of cold chain, which is the most vital component of the entire program. Keeping in view the climatic condition of Pakistan, availability of the power source and requirement of the cold chain space according to the target population at each level of hospital i.e. primary, secondary and even at tertiary care facility. Every facility must have an ice lined refrigerator, cold boxes, and vaccines carriers. Funds must be allocated for regular repair and maintenance of the cold chain equipment. Periodic replacement and upgradation of the cold chain equipment is also a requisite that would require appropriate funds allocation.

2. *Program governance, management and accountability*: The role of the federal ministry of health and federal EPI cell in the overall coordination of immunization services in the country is pivotal. Forums for 'interprovincial coordination' and 'donor coordination' must be established. Program review meetings held regularly at the federal, provincial, district and health facility levels may help to improve governance of the program. Involving the private sector can also resolve some governance issues. Furthermore, participation of local organizations, community leaders, and volunteers can provide timely feedback to improve the immunization services.

3. *Capacity building and human resource*: A fresh review and mapping of the EPI HR and their capacity is required for chalking out a plan for an in-service training. This exercise will bring to light the HR gaps at the federal and provincial EPI cells, and will lead to

recruitment of new vaccinators and women volunteers at the community level. This may help to reduce workload on the existing staff, and perhaps task shift to some extent. Strategies for capacity building may encompass short courses on public health epidemiology, different operational aspects in immunization for the mid-level managers, vaccinators and supervisors. All newly recruited vaccinators must undergo intensive 3 months practical training before being authorized to administer EPI injections independently. This is equally important for the Lady Health Workers who assist the EPI in national immunization days. In addition to this, refresher trainings must be arranged at least once every 2 years. The EPI management staff should undergo management trainings before assuming immunization program responsibilities at any level. This should be followed by a refresher training at least once every 2 years.

4. *Immunization policy and legislation for service delivery*: Private sector, which is the first contact of care seeking for 80% of the population in Pakistan and which is perceived as more trustworthy, must also be engaged for the delivery of routine immunization. This engagement will have the potential to improve access as well as coverage. Likewise, if task shifting to LHWs is required, legislation and policy decisions must be taken expeditiously. The program needs a clear strategy on immunization through outreach as well as fixed centers. Integration of EPI with other public health interventions such as breastfeeding, maternal nutrition, community midwifery, micronutrients etc. must be considered. A common integrated program (with common funding) will allow all health workers of various units to take up immunization related activities as their responsibility. For instance, a health staff counseling a mother for breastfeeding or nutrition must also inform her about importance and schedule of immunization. Workforce shortage in EPI will thus be addressed, and more workforce will be propagating the EPI messages and will be available to deliver services.

5. *Information systems*: EPI data reliability ought to be enhanced through a critical review of the current reporting system and by objectively examining the procedures, roles and responsibilities, and also the reasons for its under-performance. Employing newer technologies (i.e. GPS, tablets, smart phones etc.) can potentially improve the timeliness and accuracy of the data.

6. *Engaging communities*: Campaigns for demand creation need careful planning and coordination with communication experts. Increase in the allocation of funds for mass campaigns, and to the districts to customize messages in their local context is needed. Developing a deeper understanding of locally held perceptions or misperceptions that shape the behaviors of the community will be helpful in certain geographical areas that have historically proved resistant to EPI efforts. Face to face communication and advocacy with local opinion leaders and community elders should be continued. Such engagements with communities have shown to be fruitful for increasing the coverage of immunization.

7. *Risk analysis*: Periodic assessment of the high risk, high priority districts and mapping of vulnerable populations must be carried out. Similarly, profiling of HR and logistic gaps is imperative. Real time information using cell phone technology can be assimilated in a dashboard where monitoring teams should instantly pick up the shortage of HR or any

supplies/logistics, and action can be taken immediately. Timely and correct interpretation of risk analysis is vital for designing context specific interventions. Community's role in diseases surveillance must also be tapped for early case detection and reporting, initiating an immediate response, and improving outcomes.

4. Conclusion

This chapter has endeavored to unravel a multifactorial picture responsible for insufficient immunization coverage in Pakistan. Current evidence suggests that focusing on governance of the program, improving facility-based service delivery and addressing community perceptions could result in the biggest payoffs. Within a multi-cultural milieu and a complex health system, the country presents an ideal case for embarking upon more systematic health systems and implementation research to develop an empirical evidence base and to re-build the routine immunization program to serve the people who are most in need. Moreover, research conducted in the universities must be communicated in simplistic manner to the implementers and policy makers. The current situation pleads the case for generating fresh evidence in order to review policy, programmatic approach, service delivery and stakeholder engagement for improving EPI.

Acknowledgements

Not applicable.

Conflict of interests

None.

List of abbreviations

EPI	Expanded Program on Immunization
WHO	World Health Organization
LMIC	lower middle income countries
BCG	Bacillus Calmette–Guérin
UNICEF	United Nations Children's Emergency Fund
GAVI	Global Alliance for Vaccines & Immunization

JICA	Japan International Cooperation Agency
HR	human resources
NGOs	non-governmental organizations
LHW	lady health workers
DHIS	District Health Information System
GPS	global positioning system

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Chapter 6

Preventing Vaccine Failure in Poultry Flocks

Aamir Sharif and Tanveer Ahmad

Additional information is available at the end of the chapter

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Abstract

Poultry sector is very useful for humans in terms of production of food items like meat and eggs. Pakistan has a developing poultry sector and is the second important sector after the textile industry. The poultry sector is encountered with many challenges; among them is the high incidence of disease outbreaks that result in colossal economic losses. The diseases of commercial and rural poultry include Newcastle disease (ND), infectious bursal disease (IBD), fowl pox, Marek's disease, infectious bronchitis (IB), avian influenza, hydropericardium syndrome, etc. The disease outbreaks have also occurred in vaccinated flocks. Better understanding of the causes of vaccine failure will result in identifying prophylactic measures regarding disease outbreaks in poultry flocks. This chapter overviews the common causes of vaccine failure and further highlights the procedures for successful immunization.

Keywords: immunization, vaccine failure, poultry

1. Introduction

Poultry sector is the source of animal proteins in the form of meat and eggs. The total strength poultry in Pakistan is 1210 million. The poultry sector provides 1,391,000 tons of meat and 18,037 million eggs annually. The contribution of poultry sector in agriculture and livestock sectors is 7.5 and 12.7%, respectively, while its contribution in total GDP is 1.4. The annual growth rate of poultry sector is 5–10% in the country. The poultry meat contributes 32.7% of total meat production in the country [1]. The demand of poultry meat has increased over the years due to the increasing demand of quality food in the form of meat and eggs.

Poultry birds being living creatures are prone to infections. Diseases are a cause of high economic losses to poultry farmers [2]. In developing countries, poultry diseases are a cause

of very large economic losses to poultry industry [3, 4]. Among bacterial, viral, parasitic and fungal diseases, the outbreak of viral diseases can cause havoc to the poultry industry causing reduced meat and egg production. The important viral diseases of poultry include Newcastle disease (ND), avian influenza, infectious bursal disease (IBD), infectious bronchitis (IB), etc. The high prevalence of diseases creates major constraints in the development of poultry sector. Immunization is the use of a biological preparation in the form of vaccine for enhanced immunity and prophylactic measures against specific diseases [5]. The process of injecting the vaccine in the body is known as vaccination. Proper vaccination can prevent losses due to diseases in poultry flocks [6]. Mostly, the vaccines are carried out against viral diseases but vaccines against salmonella, mycoplasma and coryza infections are also available. The vaccines against parasitic infections like coccidiosis are also being tested in different countries.

Vaccination is one of the most important tools for preventing diseases and in reducing the economic losses of the poultry producers [2]. Vaccination comprises the use of attenuated, killed, or recombinant organisms for stimulation of the body's immune response that recognizes the injected organism as a foreign antigen, resulting in clearing the antigen and developing memory cells in the body. Vaccination is the cheapest, reliable, effective, economical, affordable and suitable alternate for prevention of diseases in poultry flocks [5].

Live vaccines comprise a virulent virus whose pathogenicity has been weakened through consecutive cultures in living cells but the virus maintains its immunogenic antigenicity for stimulating the body's immune response; this whole process is commonly known as attenuation [7]. Commonly used live vaccines against diseases of poultry are Newcastle disease, infectious bronchitis, infectious bursal disease, etc. [8].

Killed vaccines comprise viruses whose pathogenicity has been inactivated through the use of physical and chemical means, but the protein coat structure has been maintained, which acts immunogenic. The viruses are physically inactivated by the use of ultraviolet radiations and heat and through chemical means by the use of formalin [9]. Killed vaccines against Newcastle disease and Avian Influenza are being used and have an advantage of providing long term immunity to flocks.

Vaccine failure is the consequence of the inability of the chicken to develop adequate immunity after immunization or susceptibility of bird to field outbreak after administration of vaccine [3]. High rates of 53.5% of vaccination failures have been recorded in vaccinated poultry flocks. Rates of 25.6, 25.6 and 2.3% of vaccine failure in Newcastle disease, infectious bursal disease (Gumboro) and fowl pox, respectively, have been recorded [2]. The common breaches in transportation, handling, storage and administration of vaccines are responsible for high rates of vaccine failure in poultry flocks in developing countries [2].

2. Causes of vaccine failure

The causes of vaccine failure can be categorized into two major factors: antigen factor and host response.

2.1. Antigen factors

The protective vaccine antigen is of prime importance in the production of effective vaccine. The vaccines available in the market may have the following shortcomings resulting in vaccine failure.

2.1.1. Improper formulation of vaccine

The vaccines are manufactured in a processing plant where the titer of antigen of specific virus or bacteria may not be maintained properly; as a result, the inoculums may not initiate protective immune response in birds. The titer of antigen in the vial of vaccine may be low which results in low immunity level in birds. The dose–response relationship among the virus content, serological response and clinical protection has been reported [10]. Virus concentration has a significant effect on immunogenicity of vaccines [11]. The inadequate procedure of formulation of vaccine and lack of standard procedures of vaccine formulation result in the production of nonpotent vaccine.

2.1.2. Nonusage of local antigens

Some of the viral diseases of poultry like infectious bursal disease and salmonella have many serotypes. Some of the serotypes are prevalent in one area, while others are prevalent in other areas. The local disease causing agents in any area are of prime importance for vaccine manufacturing. The strains of viruses differ from area to area. The local serotypes and locally isolated antigens are considered the most suitable immunogens for formulating vaccines. The nonusage of local vaccine antigens may result in disease outbreaks [2]. The foreign vaccine may be made from serotypes that are different from field strain [12]. Moreover, vaccination with foreign vaccine may not provide immunity to birds if the field strain is of higher virulence and of a different nature [13].

2.1.3. Improper storage temperature

After the formulation of the vaccine, its storage is of utmost importance. The freeze-dried vaccines require freezing temperatures, while lyophilized vaccines may be stored at 4°C, and during transportation the low temperature might not be properly maintained. The Marek's disease vaccine is stored in liquid nitrogen at very low temperatures, while live vaccines of ND, IBD, IB, etc. are stored at 4–8°C. The oil-based vaccines may be stored below 8°C. In the poultry sector, almost all the vaccines available are thermolabile in nature. The maintenance of proper cold chains and storage temperature is a prerequisite for optimal potency of vaccines. The shortage of electricity, weak, nonfunctional, obsolete and repaired storage equipment, high temperature during transport, refrigerators without thermometers, etc. are the common problems of vaccine storage of developing countries like Malaysia, India, Tanzania and Pakistan [14–18]. Data have been recorded about use of vaccines after purchase from the market in Nigeria, and it has been found that 16% of farmers do not perform vaccination on the date of purchase of vaccine and 7% of farmers store the vaccine on the shelf without proper preservation, thus resulting in vaccine failure [2].

2.1.4. Exposure to direct sunlight

It has been documented that vaccines are transported like ordinary drugs [2]. Direct sunlight has UV radiations which are lethal for live viruses. The exposure of vaccine to direct sunlight results in the killing of antigens present in the vial, and as a result, the number of viral antigens is reduced in the vaccine and the vaccine may become ineffective.

2.1.5. Use of expired vaccines

The potency of vaccines is maintained to a certain period of time, provided that the transportation and storage temperature is properly maintained. The use of vaccines after the date of expiry may not result in optimal immune response and can also result in vaccine failure.

2.1.6. Mutation of viruses

Some of the viruses like the influenza virus are of a mutating nature and as a result pose a serious threat regarding the effectiveness of vaccine against certain diseases.

2.2. Host factors

The poultry birds to be vaccinated against diseases may not respond effectively against vaccines due to the following shortcomings, thus resulting in vaccine failure.

2.2.1. Stress on birds

Stress is a condition of vulnerable homeostasis and is affected by management and environment factors. Birds normally have limited resources in the body for growth, response to environment changes and maintain a defense system for diseases. The stress on birds can be due to a number of factors including cold stress, heat stress, high humidity, transportation stress, intensive farming, high stocking density, overcrowding, low per bird space, decreased ventilation, poor litter conditions, accumulation of bad smell in sheds and poultry houses, off feeding, water deprivation, poor management, bad sanitary conditions, very wet or extremely dry litter, dusty environment, parasitism, nutritional deficiency, fever, and so on. In these cases, there can also be vaccine failure in livestock. The poultry birds are sensitive to both cold and warm weather [3]. Heat stress is an important factor of economic loss for the producer [19], while cold stress modifies the immune response of broilers [20]. The symptoms of stress in birds include panting, increased thirst, reduced appetite, reduced egg production, decreased weight gain, small sized eggs, thin egg shells, reduced growth, prostration, etc. All the factors including management conditions, substandard hygienic conditions, etc. contribute to the possible causes of high economic losses by leading to vaccine failure [21].

2.2.2. Concurrent disease

It is highly important that the vaccination should be done in healthy birds. The vaccination in sick and diseased birds may not provide fruitful results; rather, vaccine reaction may occur leading to extra stress and an increased morbidity and mortality rate. Moreover, any other

disease condition may also contribute to vaccine failure. When the birds are morbid due to the same disease for which vaccination had been done, then there will also be vaccine failure because the antibodies produced against the pathogenic agent will neutralize the antigen of vaccine and a reaction may take place in the body of birds and vaccination may worsen the condition of disease.

2.2.3. Immunosuppressive diseases

Certain diseases are immune-suppressive in poultry flocks like mycotoxicosis, infectious bursal diseases (Gumboro), chicken infectious anemia, Marek's disease, etc. These immune suppressive diseases may also lead to vaccine failure. The fungal toxins present in poultry feed have a bad effect on the feed conversion, growth, health and immune status. The fungal toxins cause the following effects: carcinogenic, allergic, hypersensitivity and depression. The common age of infection of infectious bursal disease (Gumboro) in poultry flock is at 3rd to 7th week of age. The bursa is a lymphoid organ in poultry where maturation of B cells takes place in poultry. The infection of IBD during this stage of age may lead to permanent damage to bursa; as a result, the maturation of B Cells may not take place in birds throughout their life span and thus the birds remain prone to vaccine failure during the rest of their lives.

2.2.4. Immaturity of birds

The receptors for some antigens develop in the body with advancing age. Some of receptors of virus develop as early as with the hatch of a chick. The receptors of diseases like Newcastle disease, infectious bronchitis, etc. develop at a very early age while the receptors of diseases like infectious bursa disease, fowl pox, etc. develop late in the body. Vaccination at a very early age before the development of certain receptors may also result in vaccine failure. The age of the bird is very important at the time of vaccination.

2.2.5. Interaction with maternal antibodies

The antibodies of certain viral diseases are transmitted through eggs. As the breeder/parent flocks of poultry are routinely vaccinated against viral diseases which are prevalent in the area, the newly hatched chicks have maternal antibodies in their blood and these can interact with vaccine antigens. The antibodies against ND virus and IBD virus are transmitted in eggs and provide protection to the newly hatched chicks during the first week of birth. High maternal antibodies interfere with multiplication of live vaccines and reduce the level of immunity production in the chicks. The use of live vaccines during the first week of birth in chicks against diseases whose maternal antibodies still persist in the body of the chick will result in neutralizing of antigen and active immunity may not be provided by the vaccine [22].

2.2.6. Improper route of administration

The vaccines have specific routes for their administration in the body of the bird, that is, through oral, subcutaneous (S/C), intramuscular (I/M), wing web (W/W), drinking water (D/W), eye dropping (E/D), spray, etc. Not following f specific recommended routes of

vaccination may result in vaccine failure in poultry flocks. The fault of administering the vaccine also results in vaccine failure [3, 4].

2.2.7. Inadequate dosage

If the optimum dose of vaccine had not been injected in the bird, then there is also failure of vaccine. Overdosage may lead to reaction, and underdosage can lead to vaccine failure. There are certain factors which cause reduction in optimal vaccine dosage, that is, use of chlorinated water for vaccination, use of water having antimicrobial contents, etc. Moreover, in the case of injecting vaccine to more number of birds than recommended by the company or manufacturer, the low dose will be available to the whole the flock and thus may be prone to low vaccine titers and vaccine failure.

2.2.8. Lack of booster dose

Some of the vaccines require a booster dose for successful immunization. The booster dose is required after 10–20 days of the initial dose. The initial dose is required for priming of vaccine while the booster is required for maximum protection against antigen. The lack of booster dose results in low antibody titers, resulting in vaccine failure.

2.2.9. Wrong timing of vaccination

Mostly the vaccines should be done early in the morning or later if it is during summer. The birds feel comfortable during cold hours of the day. As a result, a good response is obtained after vaccination. Otherwise, the chances of vaccine failures are increased in the case of vaccinating birds during the hot hours of the day.

2.2.10. Climatic factors

The climate variation is a change in climatic parameters (temperature, rainfall, humidity and soil moisture) [23]. Climate change affects both living and nonliving creatures, thus contributing to the health of poultry flocks and may lead to vaccine failure and disease outbreaks.

3. Preventing vaccine failure

The following procedures can prevent vaccine failure in livestock and poultry flocks.

3.1. Vaccine factors

3.1.1. Proper formulation of vaccine

The vaccines must be properly formulated. The dosage of vaccinal antigen and properly processed vaccines provide good results and prevent vaccine failure. The record of all batches of

vaccines and their standard tests of vaccine potency may be maintained. Moreover, the titer of antigen should be optimal so that the proper immunity level may be provided by the vaccine.

3.1.2. Use of local strains of viruses

For maximum immune protection, the local strains of antigens must be used for manufacturing of vaccine. The local disease causing agents of any area are specific targeted pathogens and antigens from local disease outbreaks and provide maximum protection against local disease causing organisms.

3.1.3. Adequate procedure of vaccine formulation

The viruses used for vaccine production are harvested in live cells like chicken embryo. The bacteria used for vaccine production are culture in growth media like nutrient agar, etc. Similarly, the procedures for live attenuated, killed inactive vaccines, subunit vaccines differ from antigen to antigen. The adequate procedure for vaccine formulation will result in a maximum immune response from the antigen and hence a successful immune response.

3.1.4. Proper storage and cold chain temperature

Vaccines are to be manufactured in a plant and then after stored and transported to remote areas. Temperature has direct effect on the efficacy of vaccine [24]. The vaccines lose their potency with the passage of time; hence, they require proper cold temperatures to remain stable and viable for long periods of time. The proper storage and cold chain temperature of vaccine is of utmost importance; the vaccines must be stored below 4°C. The storage of food items, chemotherapeutic agents, specimens for pathological examinations, tissue samples for laboratory findings along with vaccine should be avoided [25]. During transportation, the maintenance of cold chain is a challenge for developing countries. A number of factors create hurdles in maintaining cold chain systems including loss of electric power, substandard refrigeration system, overchilling, etc. Moreover, the extra chilling of oil-based vaccines results in crystal formation of adjuvant material of vaccine like aluminum salts, etc. resulting in reduced potency of vaccines. The thermostable vaccines can be stored at 2–8°C and has more significance where cold chain temperature is not maintained and is less expensive [24]. Thermostable vaccines have some resistance to cold and hot environments, while freeze-dried vaccines should be preserved and stored at low temperatures in the refrigerator at 4°C and even during the transport of vaccine the cooling/ice blocks should be used to maintain low temperatures during transportation of vaccine. Freezing and thawing must be avoided. The vaccines must only be brought out of the refrigerator/freezer at the time of use at the farm. The live vaccines in poultry flocks must be used within 2 h of its reconstitution. Once they have been reconstituted, they drop their potency rapidly. The reconstituted vaccines should be used as early as possible and unused vaccines may be stored in the refrigerator for a maximum of 6 h; after that period the vaccines should be discarded.

The use of thermostable vaccines can be an alternative to overcome the difficulties related to cold chain and storage temperature [26]. The thermostable vaccines can maintain their

potency and vaccinal activity for 1 year at 2–8°C and for 3 months up to 28°C in dried form [27]. Routes including intraocular, intranasal, paternal (injection) and oral (drinking water and feed) can be used for administration of thermostable vaccines [28, 29].

3.1.5. Avoiding exposure to direct sunlight

Direct exposure of sunlight results in the killing of antigen present in the vial; as a result, the titer of vaccine antigens are reduced in the vaccine and it may become ineffective. During formulation of solution for oral or parental vaccines, direct exposure to sunlight should be avoided and for oral vaccines the cap of the vaccine vial should be opened inside water. The vaccines should be mixed in drinking water in a room or in a shady place; moreover, during the transportation of vaccine, black or colored bags and cartons should be used to prevent sunlight affecting the vaccine.

3.1.6. Avoiding use of expired vaccines

The date of expiry mentioned on the vial of vaccine should be checked before opening the vaccine vial. The expired vaccines should be discarded or returned to the manufacturers. At places where vaccines are frequently used, it should be a practice to purchase a fresh stock of vaccines. Some oil-based vaccines have a very narrow range of shelf life of 3–6 months. While some lyophilized live vaccines have longer shelf life of 1–2 years, provided the vaccines are stored at proper temperatures. The use of expired vaccines should be avoided [25].

3.1.7. Use of adjuvant

Adjuvants are substances that are added in the vaccine to increase the bioavailability of vaccine. In the poultry sector, the oral route of vaccination is followed for most of the vaccines. The oral route of vaccine delivery is difficult due to barriers in the gastrointestinal tract. In order to overcome this challenge, the antigen must be protected from such an environment and the immune response must be activated. This challenge can be overcome by the use of adjutants. Adjuvants improve the safety of the vaccine and have a potential effect on inducing mucosal immune response [30]. The use of adjuvants provides good results for live vaccines. The adjuvants enhance the availability of vaccine and act as a sticking agent for vaccine and the mucous membranes of the body.

3.1.8. Use of stabilizers

Stabilizers are substances which are added in a vaccine to increase the shelf life of the vaccine. The stabilizers like Vac-Safe (Intervet), Vital Blue, etc. can be used for oral live vaccines like ND, IBD, IB, etc. of poultry. The skimmed milk at the rate of 2 g/L can also be added as a suitable alternative stabilizer [31].

3.1.9. Manufacturer guidelines

The company's manufacturing guidelines provide valuable information regarding vaccine efficacy, usage, storage and route. The guidelines are: (1) open the vaccine vial in water and (2) use one complete vial after opening it. The vaccines must be utilized as early as possible

after its reconstitution in diluents, etc. Once the vaccine is reconstituted, the time limit is set. Vaccines must be used within 2 h of their reconstitution during winter and within 1 h of their reconstitution during summer. IB vaccines lose potency after 1 h of their reconstitution, while pox vaccines lose 50% of their potency after 1 h of their reconstitution [32].

3.1.10. Use of immune boosters

There are many substances that have been used in poultry for immune stimulation. Some of them are vitamin E, selenium and levamisole [33, 34]. The selenium supplementation has effect on enhancing humoral immune response in chicks [35, 36]. The selenium supplementation increased natural resistance of increasing response of organisms to antigenic stimuli [37, 38]. The increased humoral antibody titers are observed when selenium is used in feed [39].

3.1.11. Booster dose

Some of the vaccines require a booster dose for successful immunization [2]. The booster dose is required after 10–20 days of the initial dose. The initial dose is required for priming of vaccine, while the booster is required for maximum protection against antigen. The lack of booster dose results in low antibody titers. As a result, vaccine failure may result. It has been documented that the priming with live attenuated vaccine followed by booster of killed vaccine and second booster with live vaccine provides best protection against Newcastle disease [40]. However, subsequent inoculums are also required at regular intervals.

3.2. Host factors

An effective vaccine response may be obtained if the bird is healthy. The following recommendations/guidelines can overcome the shortcomings regarding prevention of vaccine failure.

3.2.1. Stress-free birds

All types of stresses mentioned earlier should be avoided before administration of vaccines to poultry birds. The temperature of the environment and sheds should be normal before vaccination. Moreover, the birds should be in a good physical condition before administration of vaccines. Stress suppresses the chicken's immune response, and during these conditions of stress, birds should not be vaccinated [41]. The stress on birds can be minimized by the use of vitamins and minerals in drinking water before, during and after vaccination [13].

3.2.2. Deworming before vaccination

The adult birds may be dewormed before vaccination at least 15 days before injection of vaccine; moreover, diseased birds should be treated properly and be given vaccines after recovery. Only healthy flocks should be vaccinated.

3.2.3. Monitoring of subclinical infections

Some of the diseases in poultry have subclinical infections, like coccidiosis. The birds apparently seem health, but subclinical infections persist in birds over long periods of time, which

have previously been infected with coccidiosis infections. On the day of vaccination, the birds should be closely monitored. The apparent health of flock should be analyzed. Moreover, the color and consistency of fecal droppings, abnormal sounds from birds, respiratory distress, etc. should be evaluated. After being satisfied with the proper health status of the bids, the concerned staff may be allowed to vaccinate the flock.

3.2.4. Balanced feed

Nutrition plays a significant role in the development and function of the immune system [42]. The commercial feed offered to poultry should be analyzed regularly and the level of toxins be checked on a regular basis. Especially in summer and humid environment conditions, the fungus grows on feed ingredients and fungal metabolites gain entry into the body of poultry, and as a result, they cause immune-suppression, decreased growth, hypersensitivity and decreased feed intake.

3.2.5. Maturity of bird

In poultry birds, age is considered for the vaccination of bird; receptors for different pathogens develop in the body of poultry bird at specific ages, so the vaccination is done keeping in view the age of the bird, that is, ND + IB vaccine is done on the first day of birth. Similarly, the Marek's disease vaccine is done immediately after hatching of chicks in the hatchery machine. The IBD (infectious bursal disease/Gumboro) vaccine is done at 10–12 days of birth and booster is given after 10 days. In broiler birds, the hydropericardium syndrome (HPS)/ Angara vaccine is done at 21–23 days of birth. So, the age of the bird is very important for vaccination. The domestic/rural poultry requires injection of ND after every 2–2.5 months.

3.2.6. Consideration of maternal antibodies

As the breeder/parent flocks of poultry are routinely vaccinated against viral diseases which are prevalent in the area, the newly hatched chicks have maternal antibodies in their blood. It is suggested that the bird should be a minimum 11 days old at the time of administration of IBD vaccine and 7 days old at the time of administration of ND vaccine [31].

3.2.7. Proper vaccination schedule

In Pakistan, the outbreak of diseases like infectious bronchitis and avian influenza occurs in birds during winter; for that purpose, the birds must be vaccinated prior to winter, so that proper antibody titer may be reached in birds before exposure to the disease causing virus or bacteria in birds. To avoid any economic loss, a record may be maintained and a strict vaccination schedule according to disease prevalence in the area must be followed in poultry flocks [43].

3.2.8. Preparation of flocks for vaccination

The poultry flocks are properly prepared for administration of oral vaccines. The birds are offered feed and are kept off water for 2 h before administration of vaccines. The drinkers

are properly washed and the vaccine is given to birds. The number of drinkers is increased in order to ensure that all the birds drink vaccine water. The water should be provided to birds in such a way that birds drink all the vaccine water within 2 h. The birds are regularly moved during this process so that all the birds drink water containing the vaccine virus. The stabilizers and coloring agents can be added in the vaccine. A tinge of the coloring agent can also be noticed on the beak of birds which indicates the drinking of water.

3.2.9. Host resistance

Certain genes are discovered which have genetic resistance against viral diseases of poultry [44]. The breeding for disease resistance may provide good long-term solutions for disease control [45]. However, the emergence of new genetic groups and mutations require new vaccine practices for successful immunization [46].

3.2.10. Vitamin and mineral supplementation

Vitamin and mineral supplements help to develop immune response by acting on the immune cell or by changing metabolic or endocrine functions [47]; as a result, the antibodies are produced in the body at a faster rate and a protective level of antibodies is gained in a shorter time. Vitamin E and selenium have a role in modulating the immune response and have shown good results in preventing vaccine failure. Research conducted shows that vitamin E may enhance immune response to antigens in cockerels but excessive vitamin E may depress specific immune responses [48]. Administration of excess vitamins, amino acids, minerals and their combinations enhance the disease resistance by stimulating humoral and cellular immunity and phagocytosis [49]. Optimal vitamin nutrition is required for optimal immune response and disease resistance. The addition of higher levels of vitamin A, C, E and Selenium ensures better immune response of birds to vaccination and reduces the chances of vaccination failure in broiler poultry flocks [50]. Studies have suggested that the nutrient levels that are adequate for growth and feed efficiency may not be adequate for normal immunity for maximizing the resistance to disease [51, 52].

3.2.11. Continuous surveillance

The regular and continuous surveillance of prevalent diseases should be conducted in order to collect data on the disease pattern. For this purpose, the blood/serum and fecal samples may be collected and sent to the laboratory for diagnosis of disease. Moreover, the tracheal and cloacal swabs can also be sent to the laboratory for isolation of pathogens. The antibodies titer against injected vaccines may be got routinely checked for maintaining optimum titer of antibody against the disease.

3.2.12. General precautions

Considerations regarding the use of live and killed vaccines should also be kept in mind during vaccination. The live vaccines may cause vaccine reactions and injection of killed vaccines may cause local tissue reactions. Therefore, only an expert professional or qualified veterinary assistant should be allowed to vaccinate the poultry birds. In general, vaccination should be

done during early hours of the day or late hours after noon during summer. Vaccination during hot hours of the day may not give good results. Moreover, after transportation of birds, the birds should be given proper rest before vaccination [2].

4. Conclusion

Vaccination may be considered as insurance against diseases. A successful vaccination program is dependent on many factors including vaccine handling, quality and nature of vaccine, use of local antigens, immunogenic response inside the body of the bird and following manufacturers' instructions. Although the disease outbreaks against specific diseases in nonvaccinated flocks cause very high economic losses, the severity of disease outbreaks in properly vaccinated flocks is low. The potential threat of disease outbreaks even in vaccinated flocks cannot be avoided 100%, but the losses can be minimized through thoughtful consideration of success of vaccination program for poultry flocks. In Pakistan, there is a high incidence and prevalence of contagious diseases of poultry and vaccination is the only tool to prevent birds from diseases. Through preventing vaccine failure, the productivity of food items like meat and eggs can be increased in the country, and shortage of animal protein can be overcome and thus per capita availability of eggs and meat can be increased. Moreover, the poultry sector can play a better role in the economy of the country by decreasing economic losses due to vaccine failure, thus increasing annual share in GDP value and becoming a major contributor of the agricultural sector of the country.

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Chapter 7

Practical Finance Strategies in Immunization

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Additional information is available at the end of the chapter

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Abstract

My first goal is to present the basic immunization problem (BIP) as it is understood in finance. BIP relies on a construction of such a bond portfolio (BP), meaning a selection of individual bonds, that the single liability to pay L dollars q years from now will be discharged by means of BP (a patient will return to health at time q), no matter what random shift $a(t)$ (a particular disease) will occur in the future. What kind of a function is a shift of interest rates is critically important because both present and future values of BP depend solely on underlying interest rates. Having identified shifts (diseases) against which a BP is immunized, the natural question arises how to find among such immunized (immune) portfolios the best ones. In the context of finance, it means bond portfolios with maximal unanticipated rate of return. My second goal is to trigger interest among medical scientists by suggesting that certain finance notions, such as duration and convexity of a bond portfolio, might give extra insight to medical researchers working in the immunization area both into BIP and into similar problems in medicine. A considerable attention is also paid to certain mathematical notions (base of a linear space, a Hilbert space, triangular functions) because of their successful applications to problem-solving occurring in bond portfolio immunization.

Keywords: immunization, immunity, active immunity, passive immunity, best immunization strategy, duration, convexity, barbell immunization strategy, focused immunization strategy

1. Introduction

In this chapter, I present one of the research areas existing in finance called bond portfolio immunization (BPI). My goal is to make it known to medical researchers dealing with immunity (resistance) of human organisms to diseases. I feature not only basic notions, problems,

and solutions occurring in BPI, but also selected mathematical concepts and tools which proved to be instrumental in developing BPI. I do believe that such information has a good chance to be useful in creation of immunity against particular diseases. Bond investors are called immunizers if, possessing C dollars today, they must achieve an investment goal of L dollars q years from now (a human organism or a particular human organ must achieve a certain level of health q years from now); here L is the future value of C at time q under the current interest rates. This investment goal must be accomplished by means of an appropriately selected bond portfolio, even despite unfavorable sudden change (shift) in interest rates (appearance of a disease), having in mind that the present and future prices of all bonds depend solely on interest rates.

Although, as it will be demonstrated in Sections 3.1, 3.2, and 3.3, immunization against all shifts is never possible, there are many results giving sufficient, or necessary and sufficient, conditions for immunization against a certain classes of shifts (certain diseases). It is worth to know that in the financial immunization, there is no such thing as *acquired* immunity (immunity that develops in a human after exposure to a suitable agent) and *active* immunity (acquired through production of antibodies within the organism in response to the presence of antigens).

Such types of immunization might theoretically take place on a bond market only if a bond holder had the right to change the coupon payments, which is completely out of the question. In other words, the immunization in financial reality has features of *passive* immunity, being in fact a short-acting immunity. On the other hand, however, a BP manager can achieve the state of a BP being all the time immune against a specific class of shifts, provided the manager regularly (every week or so) performs (if necessary) subsequent adjustments of his/her BP according to their expertise in the area of immunization theory.

Theorem 1 (Section 2.4.2) as well as Theorems 3 and 4 allows one to look at immunization from a different perspective. They enable one to identify all shifts a(t) (diseases) of the term structure s(t) of interest rates against which BP is already (fully) immunized, that is, protected against loss of its value at time q. Finally, having identified immunized (immune) bond portfolios, the natural question arises how to find among them the best ones. This topic is dealt with in Section 4.

Below, I shall present (i) what are bonds and bond portfolios; (ii) what is meant by standard (and general) immunization problem; (iii) historical development of immunization theory; (iv) overview of some recent results; (v) the concept of a Hilbert space, and a base in a linear space; (vi) application of orthogonal polynomials to description of the class IMMU of all shifts (diseases) against which a given bond portfolio is immunized; (vii) triangular functions as a base for the linear space IMMU; and (viii) the crucial role of the notions of duration and convexity in choosing the "best" immunized (immune) bond portfolios.

2. Immunization in finance

Below, we will introduce the concept of bonds, formulate the standard and general immunization problem, and outline the development of immunization theory in finance, from the beginning to the latest achievements.

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2.1. What are bonds?

Each bond with a face value (par value) of F dollars is a financial instrument which generates to its buyer (holder) specified payments every 6 months (or every quarter, every year) in the form of coupons, plus par value paid only at the termination (maturity) of the bond. The face value represents the amount borrowed by the seller (issuer) of a bond from the bond buyer. The coupons represent a predetermined percentage, say 3%, of face value F; if F = $10,000, then all coupons paid per year sum up to $300. Each bond has its own life span (maturity) of n years (3, 5, 10, 20 years, etc.)

A bond portfolio (BP), by its definition, is a collection of different bonds with various maturities. Thus, each BP generates a more complicated cash flow pattern than a single bond does. A cash flow generated by a BP consists of various size payments c_i, $1 \le i \le m$, (coupons and par values generated by all kinds of bonds forming that portfolio) at certain dates $t_1, t_2, t_3, \ldots, t_m$ from an interval $[t_0, \mathrm{T}]$, where t_0 is the date when BP was purchased, while T stands for the highest maturity of all bonds tradable on a given debt market D.

The present and future value of each bond, and consequently each bond portfolio, depends solely on current interest rates $s(t)$, which in the simplest case are identical for all maturities t, that is, $s(t) \equiv s$, $t \in [t_0; \mathrm{T}]$. By the term structure of interest rates, one understands a schedule of spot interest rates $s(t)$ which are estimated from the yields (returns) of all coupon-bearing bonds. It is well known that interest rates are shaped under various random market forces.

2.2. Standard and general immunization problem

The standard immunization problem relies on a construction of such a bond portfolio with the present value of C dollars that the single liability to pay L dollars q years from now (L is the future value of C) by means of the cash flow generated by BP will be secured regardless of how adverse changes in interest rates will occur in a future. This nontrivial problem is automatically solved by each zero-coupon bond maturing at time q with par value of L dollars. Thus, using medical terminology, one may say that such a zero-coupon bond possesses an innate (natural) immunity. Unfortunately, in practice, such zero-coupon bonds rarely exist.

Besides, an investor may already possess bonds and would like to buy additional ones so that the created, in this way, portfolio BP with the present value of C dollars would secure the payment of L dollars q years from now. Having built such a portfolio, the investor would immunize (hedge) their own investment against a loss of its value at time q. We assume that the new term structure will always be of the form s*(t) = s(t) + a(t), where a(t) belongs to a certain class of shifts (diseases).

On the other hand, the general immunization problem relies on a construction of such a bond portfolio BP with the present value of C dollars that multiply liabilities to pay L_i dollars at specified instances of time will be secured by means of the cash flow generated by BP regardless of adverse changes/shifts a(t) of interest rates in a future.

2.3. Beginnings of immunization

Immunization as a concept dates back as far as to articles [1, 2]. However, not until work [3] of Fisher and Weil was the impact of interest shifts on the design of immunization strategies

rigorously studied. In a vast majority of publications, immunization was based on a specific stochastic process governing interest rate shifts a(t). In [2], Redington discussed immunization in the context of an actuarial company which had projected liability outflows L(t) at some finite number M of instances (dates) t_k and anticipated inflows $A(t_i)$ at N (typically) different dates t_i. It was assumed that interest rates were flat, that is, $s(t) \equiv s$, and shocks a(t) of interest rates s(t) meant their parallel movements, that is, $a(t) \equiv \lambda$.

In such a situation, the company's task was to choose inflows A(t) in such a manner that the outflows L(t) would be discharged if the interest rates $s(t) \equiv s$ moved to their new constant level $s^*(t) \equiv s + \lambda$. To recall Redington's main result, let us note that $V = \sum_{t=1}^{t=N} \frac{A(t)}{(1+s)^t}$ represents the present value of inflows A(t) occurring at instances t_i; a similar formula holds for liabilities L(t). Redington introduced the notion of a "mean term" having in mind the weighted average of the dates when the flows are to be received (in case of assets) or have to be discharged (in case of liabilities). This "mean term" was nothing different than the concept of *duration* introduced by Macaulay in [1]. These two authors understood duration as:

$$D = \sum_{t=1}^{t=N} tw_t; w_t = \frac{A(t)}{V(1+s)^t}; \sum_{t=1}^{t=N} w_t = 1 \tag{1}$$

where w_t tells us what portion (weight) of the entire cash flow is represented by A(t) in terms of today's money. It was proved in [2] that any *parallel* movement (shift) of the *flat* term structure $s(t) \equiv s$ of interest rates would affect the value of the assets in the same way as it would affect the value of liabilities if duration D_A of assets $A(t)$ were equal to duration D_L of liabilities $L(t)$, and additionally, the so-called *convexity* of the assets would exceed that of the liabilities.

2.4. Assumptions concerning term structure of interest rates and admissible shifts: historical development

Twenty years later, Fisher and Weil [3] restricted themselves to a single liability at a specified date q, but significantly weakened the adopted so far assumption that the term structure was flat, that is, $s(t) \equiv s$. Denoting current interest rates s(t) as h(0,t), they allowed h(0,t) to be a function of arbitrary shape with h(0,t), $0 \le t \le N$, meaning annualized returns on zero-coupon default-free bonds tradable on a debt market D. However, they upheld the strong assumption concerning the admissible shifts a(t) by supposing that h(0,t) was subject only to a random additive shift of the form $h^*(0,t) = h(0,t) + \lambda$ for $0 \le t \le N$ appearing instantly after the acquisition of a bond portfolio.

They applied (popular already at that time) continuous compounding of cash flows A(t) and L(t), which in their approach represented instantaneous rate of payments per one unit of time rather than payments themselves, so that the present value of the assets V_A could be expressed by means of an integral

$$V_A = \int_0^N A(t) \exp\left[-h(0,t)\right] t dt \tag{2}$$

while the duration D_A was given by

$$D_A = \int_0^N w_t t dt = \int_0^N \frac{A(t)\exp\left[-h(0,t)\right]}{V_A} t dt. \tag{3}$$

It was stated in [3] that immunization was secured if the duration of the assets D_A equaled the duration of the single liability to be discharged at time q: $D_A = \int_0^N w_t t dt = q$. Clearly, duration of any single inflow or outflow at time q equals q since all weights, except for the one at date q, are equal to 0 (zero).

2.4.1. Further developments of immunization theory

In subsequent 20–30 years of development of immunization theory, the strong assumption made so far that interest rates were subject to random shifts of the form $h^*(0,t) = h(0,t) + \lambda$ was being dropped. Many authors began to study shifts governed by some specific stochastic processes. For example, it was proved in [4] that some alternative stochastic processes permitted immunization, and others did not. When immunization can occur, formulas for calculating the resulting duration differ depending on the underlying stochastic process.

Few years later, it was demonstrated in [5] that these differences may be significant. For example, when a multiplicative stochastic process λ is used, that is, $h^*(0,t) = \lambda h(0,t)$ instead of additive stochastic process $h^*(0,t) = h(0,t) + \lambda$, one would obtain the implicit formula for the immunizing duration shown as Eq. (13) in [6] on p. 29. On the other hand, both the multiplicative shift

$$h^*(0,t) = \left[1 + \frac{\lambda \ln(1+\alpha t)}{\alpha t}\right] h(0,t); \tag{4}$$

and the additive one

$$h^*(0,t) = h(0,t) + \frac{\lambda \ln(1+\alpha t)}{\alpha t}, \tag{5}$$

were studied in [7], where suitable implicit formulas for the respective immunizing durations were derived.

Another approach, called contingent immunization, was developed in [8]. It consists of building a bond portfolio with a duration shorter or longer than the investor's planning horizon, taking into account "personal" expectations of a bond manger with regard interest rates. The idea standing behind such approach is to take advantage of the manager's ability to forecast interest rate movements (diseases) as long as his/her predictions are accurate.

2.4.2. Latest developments of immunization theory

The contingent immunization was implemented in many situations for various term structures of interest rates; see [9]. Other than mentioned in Eqs. (4) and (5), stochastic process governing admissible shifts was analyzed in [10].

Striving to offer a more general approach, the authors of article [11] did not confine ourselves to a specific process governing shifts, but allowed them to belong to a certain class of functions, such as, for example, polynomials of degree less than some specified number n (pp. 858–861). In this way, they did not expose themselves to any model misspecification risk, similarly as Zheng in [12].

The larger class of shifts (diseases) against which the immunization will work, the better. Having this in mind, the interval $[t_0; \mathrm{T}]$ was divided in [6] into n equal nonoverlapping subintervals I_k, $1 \le k \le n$, and set $a_k(t) = 1$ when $t \in I_k$ and $a_k(t) = 0$ otherwise (p. 34). The admissible shifts were assumed to be piecewise constant functions of the form $\sum_{k=1}^{k=n} \lambda_k a_k(t)$. The authors made a general assumption stating that a BP generates inflows

$$A(t) = A_0(t) + \sum_{k=1}^{k=m} c_k \delta(t - t_k), \tag{6}$$

with $A_0(t)$ representing an instantaneous rate of cash payout, while s_k standing for single payment at instances $t_1, t_2, t_3, \ldots, t_m$. The expression $\delta(t - t_k)$, with $\delta(t)$ standing for a Dirac delta function, was employed in order to make integration possible. The following result (Theorem 3, pp. 34–35 in [6]) was then proved.

Theorem 1. If q denotes the date when the single liability of L dollars has to be discharged by means of the cumulative value of assets *A(t)*, then the immunization is secured against all adverse piecewise constant shifts $\sum_{k=1}^{k=n} \lambda_k a_k(t)$ of interest rates *h(0,t)* if and only if

$$a_k(q)q = \int_0^T \frac{A(t) \exp[-h(0,t)]t}{V_A} a_k(t) t dt, \quad 1 \le k \le n, \tag{7}$$

where V_A stands for the present value of the portfolio represented by assets *A(t)*.

Remark 1. When $n = 1$, then Theorem 1 gives a sufficient and necessary condition

$$a(q)q = \int_0^T \frac{A(t) \exp[-h(0,t)]t}{V_A} a(t) t dt \tag{8}$$

for immunization when the term structure *h(0,t)* is subject to shifts $h(t,0) + \lambda a(t)$. In case of parallel shifts, (8) reduces to Fisher-Weil condition $q = \int_0^T \frac{A(t) \exp[-h(0,t)]t}{V_A} t dt$, which is well remembered as the following statement: Immunization is secured if the duration of the assets D_A equals the duration of the single liability.

Remark 2. Theorem 1 can also be looked at from a different perspective. Namely, one may be interested in identification of such a set of shifts a(t) of the term structure s(t) of interest rates,

say IMMU, against which a bond portfolio BP is already immunized, that is, protected against loss of its value at time q. In this context, Theorem 1 offers a sufficient and necessary condition for a shift a(t) to belong to set IMMU.

2.5. One cannot immunize against all possible shifts of interest rates development

As of today, no one was successful in building up a bond portfolio BP immunized (immune) against all shifts of interest rates (diseases). What is more, in Section 3, we demonstrate that the set IMMU is always a proper subset of all admissible shifts, being in fact a linear subspace of all shifts.

3. Overview of some recent results

In a recent paper [13], the authors found a strong evidence that momentum across various asset classes is caused by macroeconomic variables. By properly modifying their portfolio, in response to changes in macroeconomic environment, their strategy performed particularly well in times of economic distress. The obtained results allowed them to establish a link between momentum and sophisticated predictive regressions.

Aiming at securing higher effectiveness of their investment in fixed income bonds, the authors of [14] successfully used simulations of the portfolio surplus, measuring the inherent risk by means of the value-at-risk methodology. In another very recent publication [15], the authors studied immunization assuming that shifts were parallel or symmetric. A quite different approach to immunization was proposed in [16]. The authors concentrated on hedging risk inherent in bond portfolio. They divided the entire problem into two parts, by formulating a two-step optimization problem. They focused first on immunization risk, and next maximized the portfolio wealth.

In this section, it is proved that the set of all continuous shocks *a(t)* against which a bond portfolio BP is immunized is an m-dimensional linear subspace in the *(m + 1)*-dimensional linear space of all continuous shifts a(t), with m standing for the number of instances when BP promises to pay cash (coupons or par values generated by bonds forming BP). The main mathematical concept used below is the notion of a Hilbert space and the concept of a base in a Hilbert space.

3.1. When polynomials are admissible shifts

From now on, we assume that $A_0(t) \equiv 0$ in Formula 6, so that inflows given by

$$A(t) = \sum_{k=1}^{k=m} c_k \delta(t - t_k), \tag{9}$$

generate only payments c_k at specified instances $t_1, t_2, t_3, \ldots, t_m$. In such a situation, the present value of assets *A(t)* is no longer given by Eq. (2), but by

$$V_A = \sum_{k=1}^{k=m} c_i \exp\left[-c(t_k)t_k\right]. \tag{10}$$

One of two classes of admissible shifts studied in [14] was the class of polynomials.

$$a(t) = a_0 + a_1 t + a_2 t^2 + at^3 + \ldots + at^{n-1} = \sum_{j=1}^{j=n} a_{j-1} t^{j-1},\ t \in [t_0; \mathrm{T}]. \tag{11}$$

The new term structure was assumed to be of the form:

$$s^*(0,t) = s(t) + \lambda a(t),\ t \in [t_0, T], \tag{12}$$

with $a(t)$ satisfying Formula 11.

Definition 1. (see also [11], p. 859). A set S is said to be a linear space if the sum of its arbitrary 2 elements $a \in S$ and $b \in S$ belongs to S ($a + b \in S$), and for any real number r the product of r and any element $a \in S$ belongs to S as well.

The most well-known linear spaces are probably the set of all real numbers R, a two-dimensional Cartesian plane R^2, a three-dimensional linear space R^3, and their generalizations R^n, known as an n-dimensional linear spaces.

Definition 2. A set of k vectors $v^1, v^2, \ldots, v^k$ is called linearly independent if each linear combination of these vectors $\lambda^1 v^1 + \lambda^2 v^2 + \ldots + \lambda^k v^k$ is a vector different from vector 0; see Definition 2.2 (p. 860).

Definition 3. A set of linearly independent vectors from a linear space S is called a base for S if each vector $a \in S$ is a linear combination of theirs, and this property does not hold any longer after removal of any of these base vectors.

All bases have the same size and there are many of them in each linear space S. R^n is a linear space with a natural addition $x + y = (x_1 + y_1, x_2 + y_2, , \ldots, x_n + y_n)$ of two vectors, and natural multiplication $r \cdot x = (rx_1, rx_2, rx_3, \ldots, rx_n) \in R^n$ of a vector $x = (x_1, x_2, , \ldots, x_n)$ by a real number r. The most popular base in R^n is the set of n vectors: $v^1 = (1, 0, 0, \ldots, 0)$, $v^2 = (0, 1, 0, \ldots, 0)$, $v^3 = (0, 0, 1, 0, \ldots, 0)$, and so on until $v^n = (0, 0, 0, \ldots 0, 1)$. Each vector, for example, (2, 3, −7, 10), is the following linear combination of the above base vectors: 2(1, 0, 0, 0) + 3(0, 1, 0, 0) + (−7)(0, 0, 1, 0) + 10(0, 0, 0, 1).

Remark 3. The below Formula (13), being a counterpart of Formula 8, gives a necessary and sufficient condition for immunization against shifts a(t) in case when a bond portfolio BP generates payments c_k at instances t_1, t_2, t_3, …, t_m:

$$a(q)q = \sum_{i=1}^{i=m} t_i w_i a(t_i), \tag{13}$$

with weights

$$w_k = \frac{c_k \exp\left[-s(t_k)t_k\right]}{\sum\limits_{i=1}^{i=m} c_i \exp\left[-s(t_i)t_i\right]}. \tag{14}$$

Remark 4. The class of polynomials of the form (11), with fixed n, is a linear space. What is more, the subset of these polynomials satisfying Eq. (13) is a linear space, too.

Proof. Assume that a*(t)* satisfies Eq. (13). For any real number r, Eq. (13) implies $[ra(q)]q = \sum\limits_{i=1}^{i=m} t_i w_i [ra(t_i)]$ because parameters t_i and w_i remain the same. Adding Eq. (13) holding for b(t) to Eq. (13) holding for c(t), one immediately obtains the required relationship.

$$[b(q) + c(q)]q = \sum_{i=1}^{i=m} t_i w_i [b(t_i) + c(t_i)]. \tag{15}$$

Theorem 2. (see [11], Theorem 2.1). Let q denote the date when the single liability of L dollars has to be discharged by means of the cumulative value of assets (9) despite additive adverse shifts (11) (n is fixed) of interest rates s(t) so that the new interest rates will be of the form (12). Then, the subclass of shifts (11) for which immunization is secured is a *(n−1)*-dimensional linear space, denote it by IMMU, of the space of all polynomials (11), which itself has dimension n.

How to determine IMMU is demonstrated in [11] in pp. 858–860.

3.2. Continuous functions are admissible shifts: a Hilbert space approach

The other class of admissible shifts studied in [11] was the class of all continuous functions (CF) defined as always on interval $[t_0; T]$. As previously, the new interest rates (after a shift) satisfy Eq. (12) with a(t) standing this time for any CF. As previously, assets *A(t)* are given by Formula 9. It is easy to notice that the class of CF is a linear space with ordinary addition of two functions and ordinary multiplication of a function by a real number. However, it has an infinite number of independent vectors!

We shall demonstrate that the notion of a Hilbert space is very useful in the study of immunization theory. It was named after a German mathematician David Hilbert (1862–1943) who is recognized as one of the most influential and universal mathematicians of the nineteenth century and the first half of twentieth century. By definition, a Hilbert space is a linear space, say H, which is additionally equipped with so-called scalar product (a generalization of the scalar product of two vectors from R^n) defined for any two of its elements (vectors) $h_1 \in H$ and $h_2 \in H$.

A specific Hilbert space H^* of all CF (shifts) defined on interval $[t_1, T]$ was introduced in [11] and it was demonstrated that H^* had dimension m. However, the shifts of interest rates should be considered on interval $[t_0; \mathrm{T}]$ because a random and unexpected shift a(t) might appear instantly after the acquisition of BP. In such a case, the dimension of H^* would be *(m + 1)*, which is really the case. So, in this chapter, we correct and simplify the definition of a scalar product of two arbitrary continuous functions (shifts) f(t) and g(t), by letting

$$< f, g >= \sum_{k=0}^{k=m} f(t_k) g(t_k). \tag{16}$$

It is good to know that in each Hilbert space H, one can measure a distance between any two elements $h_1 \in H$ and $h_2 \in H$ by the formula $\|h_1 - h_2\|$, where $\|h\| = \sqrt{< h, h >}$ is said to be a norm of vector h. In space H^*, the norm is therefore defined as follows:

$$\|h\| = \sqrt{< h, h >} = \sqrt{\sum_{k=0}^{k=m} h(t_k) h(t_k)}. \tag{17}$$

Clearly, $\|h\| = 0$ if and only if $h(t_k) = 0$, $0 \le k \le m$. A function *h(t)* belonging to H^* is treated as an element (vector) 0 (zero) if and only if $\|h\| = 0$. Therefore, $\|h_1 - h_2\| = 0$ means, then the two functions $h_1(t)$ and $h_2(t)$ are viewed as same on interval $[t_0, T]$. It holds if and only if they coincide at all instances t_k, $0 \le k \le m$, when bond portfolio BP is paying cash.

In Theorem 3 to follow, we identify a base for H^* among polynomials. This approach is rather complicated since it involves the use of Gram-Schmidt orthogonalization procedure to determine base polynomials. In Section 3.3, a far more straightforward and easier to implement approach is presented where there is no need to identify base functions (shifts) because they are already given by Formulas (20)–(23).

Theorem 3. (compare Theorem 3.1 in [11]). Suppose a bond portfolio BP has been bought, and admissible shifts a(t) of a term structure s(t) are allowed to be continuous functions on interval $[t_0, T]$. Then, the set of these shifts equipped with the scalar product (16) is an *(m + 1)*-dimensional Hilbert space H^*, where m is the number of instances when portfolio BP generates cash. The subset of these shifts, say IMMU, against which a holder of BP is immune (will be able to discharge the liability of L dollars to be paid at time $q \in [t_0, T]$ by means of the cumulative value of assets (9)) is an m-dimensional subspace (depending to a large extent on BP) of the form

$$a(t) = a_0 P_0(t) + a_1 P_1(t) + a_2 P_2(t) + \ldots + a_m P_m(t) \tag{18}$$

where the *m + 1* polynomials $P_k(t)$, $0 \le k \le m$, constitute a base of space H^*. This base may be determined by the Gram-Schmidt orthogonalization procedure, while the coefficients $a_0, a_1, a_2, \ldots, a_m$ can be identified as solutions to the linear equation

$$[a_0P_0(q) + a_1P_1(q) + a_2P_2(q) + \ldots + a_mP_m(q)] \cdot q \cdot \sum_{k=1}^{k=m} c_k \exp[-s(t_k)t_k] = \quad a_0P_0(q) \cdot \sum_{k=1}^{k=m} c_k t_k \exp[-s(t_k)t_k] \tag{19}$$

It is worth to notice that after determination of polynomials $P_k(t)$, $0 \leq k \leq m$, all numbers $P_0(q), P_1(q), P_2(q), \ldots, P_m(q)$ are known, as well as parameters $q, c_k, t_k, s(t_k)$, so that $a_0, a_1, a_2, \ldots, a_m$ remain the only unknown variables. The readers interesting in identifying subspace IMMU are referred to Example 4.2 (pp. 863–864).

3.3. Identification of continuous shifts against which a bond portfolio is immunized: the triangular functions approach

A strict definition of triangular functions is given by Eqs. (20)–(23) below. Roughly speaking, a triangular function (sometimes called a tent function, or a hat function) is a function whose graph takes the shape of a triangle. Among our *(m + 1)* tent functions employed in this chapter, *(m − 1)* are isosceles triangles with height 1 and base 2, while the other two are perpendicular triangles with height 1 and base 1. Triangular functions have been successfully employed in signal processing as representations of idealized signals from which more realistic signals can be derived, for example, in kernel density estimation.

They also have applications in pulse code modulation as a pulse shape for transmitting digital signals, and as a matched filter for receiving the signals. Triangular functions are used to define the so-called triangular window, also known as the Bartlett window. Since they occur in the formula for Lagrange polynomials used in numerical analysis for polynomial interpolation, they are also called Lagrange functions. Their other applications include the Newton-Cotes method of numerical integration, and Shamir's secret sharing scheme in cryptography.

In the financial context, tent functions were employed in [17] for modeling shifts of the term structure of interest rates. The framework and assumptions made in this section are the same as in Section 3.2. Our purpose is to characterize the subspace IMMU of the Hilbert space H^* by means of triangular functions based on results presented in [18].

In this section, $t_1, t_2, t_3, \ldots, t_m = \mathrm{T}$ comprise not only all instances when a given bond portfolio BP generates payments, but also additionally the date q when the liability to pay L dollars has to be discharged. Below, we define $m + 1$ triangular functions $S_0(t), S_1(t), S_2(t), \ldots, S_m(t)$ whose graphs are triangles with bases $[t_0, t_1], [t_0, t_2], [t_1, t_3], \ldots, [t_{m-2}, t_m], [t_{m-1}, t_m]$. The first one $S_0(t)$ and the last one $S_m(t)$ represent perpendicular triangles, while the remaining ones are isosceles triangles.

$$S_0(t) = \frac{t - t_1}{t_0 - t_1},\ t \in [t_0; t_1] \text{ and } S_0(t) = 0 \text{ for } t \in [t_1; t_m], \tag{20}$$

$$S_m(t) = \frac{t - t_{m-1}}{t_m - t_{m-1}},\ t \in [t_{m-1}; t_m] \text{ and } S_m(t) = 0 \text{ for } t \in [t_0; t_{m-1}], \tag{21}$$

$$S_k(t) = \frac{t - t_{k-1}}{t_k - t_{k-1}},\ t \in [t_{k-1}; t_k],\ 1 \le k \le m - 1, \tag{22}$$

$$S_k(t) = \frac{t - t_{k+1}}{t_k - t_{k+1}},\ t \in [t_k; t_{k+1}];\ S_k(t) = 0 \text{ elsewhere in } [t_0; T]. \tag{23}$$

The following result is well known.

Remark 5. Each continuous function a(t) defined on $[t_0; T]$ attains the same values as the function $b(t) = a(0) \cdot S_0(t) + a(t_1) \cdot S_1(t) + a(t_2) \cdot S_2(t) + \ldots + a(t_m) \cdot S_m(t)$ (built up with *(m + 1)* triangular functions) at all points t_k, $0 \le k \le m$. Therefore, a(t) may be identified in H^* with the piecewise linear function b(t) because the distance between a(t) and b(t) in H^* is zero: ||b(t) – a (t)|| = 0.

It is a nice exercise to prove the following result.

Remark 6. The Lagrange functions $S_0(t)$, $S_1(t)$, $S_2(t)$, …, $S_m(t)$ given by (20)–(23) constitute a base for Hilbert space H^* of all admissible (continuous) shifts defined on $[t_0; T]$.

Theorem 4. The set IMMU of all shifts (continuous functions) against which a bond portfolio BP with payouts represented by (9) and the new term structure given by (12) is immunized constitutes an *m*-dimensional linear subspace in the *(m + 1)*-dimensional Hilbert space H^*.

Two examples illustrating how to identify IMMU are worked out in detail in [18], pp. 531–537. A special attention is given to continuity properties of subspace IMMU; see [18], pp. 534–537.

4. Maximizing the unanticipated rate of return among immunized bond portfolios

The natural question arises of how to select the "best" portfolios among those which are (have been) protected (immunized) against admissible shifts (movements) of interest rates? In finance, by best portfolios are meant those which yield the highest rate of return (the highest increase in the present value of a BP), resulting from a sudden shift of interest rates. Below, we present the results obtained in [19]. Rewriting a sufficient and necessary condition (13) and (14) for immunization of portfolio BP generating payouts (9), one obtains:

$$q = \sum_{i=1}^{i=m} t_i w_i v_i \tag{24}$$

and

$$v_i = \frac{a(t_i)}{a(q)} \text{ provided } a(q) \neq 0. \tag{25}$$

For each vector $v = (v_1, v_2, \ldots, v_m) \in R^m$, the class K_v of such continuous shifts *a(t)* for which (25) holds was defined in [19]. When vector $v = (1, 1, \ldots, 1) \in R^m$ is used, then the corresponding class K_v comprises all parallel shifts for which a(t) = constant. We shall call $D_v = \sum_{i=1}^{i=m} t_i w_i v_i$ the dedicated (for class K_v) duration. For zero-coupon bearing bond B_k, maturing at time t_k, the dedicated duration $D_v(B_k) = t_k \cdot v_k$ since all weights w_i, except for w_k, are equal to 0.

Theorem 5. The immunization of a bond portfolio BP against shifts a(t) from class K_v is secured if and only if $q = D_v(BP) = \sum_{i=1}^{i=m} t_i w_i v_i$.

With s(t) standing for the current interest rates, $PV[s(\cdot)] = \sum_{k=1}^{k=m} c_k \exp[-s(t_k)t_k]$ is the present value of BP. Suppose that immediately after purchasing BP, interest rates s(t) will shift to new levels s*(t) = s(t) + a(t). Then

$$PV[s(\cdot) + a(\cdot)] = \sum_{k=1}^{k=m} c_k \exp[-s(t_k) - a(t_k)]t_k.$$

4.1. Convexity of a bond portfolio

Set $C_v(BP) = \frac{1}{2}\sum_{k=1}^{m} {t_k}^2 w_k {v_k}^2$ and call it dedicated (for class K_v) convexity of portfolio *BP*; for more details, see [19], p. 105. It is easy to notice that convexity of a zero-coupon bearing bond maturing at t_k is given by the formula $C_v = \frac{1}{2}{t_k}^2 {v_k}^2$. It was proved in [19], p. 106, that so-called unanticipating rate of return resulting from a shift *a(t)* of interest rates $s(\cdot)$ is given by the formula:

$$\frac{PV[s(\cdot) + a(\cdot)] - PV[s(\cdot)]}{PV[s(\cdot)]} = -D_v(BP)a(q) + C_v(BP)a^2(q) + \sum_{k=1}^{k=m} O[a(t_k)]a(t_k)^2 \tag{26}$$

where lim O(a) = 0 when $a \to 0$. Taking into account that $a(t_k)$ are small numbers of order 0.1% = 0.001, one concludes that the third term in (26) is really very small. Since each immunized bond portfolio BP satisfies $D_v(BP) = q$, the maximal unanticipating rate of return among immunized portfolios will be achieved when dedicated convexity $C_v(BP)$ will be as high as possible.

Assumption 1. All zero-coupon bearing bonds B_k, which form a bond portfolio BP and mature at t_k, have mutually different dedicated durations, that is, $D_v(B_j) \neq D_v(B_n)$ if and only if $j \neq n$, that is, $t_j \cdot v_j \neq t_n \cdot v_n \leftrightarrow j \neq n$.

Definition 4. Following [20], p. 552, a bond portfolio BP is said to be a barbell strategy (barbell portfolio) if it is built up of two bonds, say B^1, B^2 with significantly different dedicated durations ${D_v}^1$ and ${D_v}^2$. On the other hand, BP is said to be a focused strategy (focused portfolio) if it consists of several bonds whose dedicated durations ${D_v}^j$ are centered around duration of the liability (q in our context).

Theorem 6. (see [19], Theorem 1). If Assumption 1 holds then the bond portfolio BP* with the highest unanticipated rate of return is a barbell strategy built up of zero-coupon bearing bonds B^s, B^l with minimal and maximal dedicated durations. The weights $\overline{x}_s$ and $\overline{x}_l$, expressing the amounts of payments resulting from B^s and B^l, are given by formulas:

$$\overline{x}_s = \frac{t_l v_l - q}{t_l v_l - t_s v_s}, \overline{x}_l = \frac{q - t_s v_s}{t_l v_l - t_s v_s}, \overline{x}_k = 0 \text{ for } k \neq s, k \neq l. \tag{27}$$

Comment 1. Suppose that instead of dedicated duration and dedicated convexity, we employ the classic notions of duration and convexity derived for additive shifts only. Then, $v_l \equiv 1$ and consequently Eq. (27) reduces to simpler, say classic, formulas $\overline{x}_s = \frac{t_l - q}{t_l - t_s}$, $\overline{x}_l = \frac{q - t_s}{t_l - t_s}$. The natural question arises of how much the weights given by Eq. (27) differ from the classic ones.

Finally, another interesting question arises, to what extend does the dedicated duration of the best immunized portfolio BP* differ from its Macaulay's counterpart? That is, what is the difference between $D_v(BP^*) = t_s w_s v_s + t_l w_l v_l$ and $D(BP^*) = t_s w_s + t_l w_l$ with $v_s = \frac{a(t_s)}{a(q)}$, $v_l = \frac{a(t_l)}{a(q)}$? It is easy to observe that when a shift a(t) affects the current interest rates in a similar manner at all or many points $t_1, t_2, t_3, \ldots, t_m$, then there is a good chance that $v_s \approx 1 \approx v_l$, and consequently, the difference between the dedicated duration D_v and the classic one will be very small.

For a specific situation, when shifts a(t) of interest rates s(t) satisfied the "proportionality" condition $\frac{a(t_k)}{1+s(t_k)} = \text{constant}$ (for details, see [21]), the maximal convexity and formula for the best immunizing bond portfolio was determined by means of Kuhn-Tucker conditions (pp. 139–140 in [21]). A formula for the resulting unanticipating rate of return was derived (pp. 141–142) and illustrating with an example (p. 143).

5. Concluding remarks

Let us summarize what we have said so far. Each bond portfolio BP (a human body? or a human body organ?) generates cash at various dates $t_1, t_2, t_3, \ldots, t_m$. What should (could) be substituted for cash (payments generated by a BP) in the medical setting remains an important open problem. Maybe, it is something related to a human body's performance; call this mysterious agent by Z.

In bond portfolio theory, the greater payouts generated by BP, the higher is the present value (PV) and future value (FV) of BP. An analogous statement is therefore expected in the medical context. Having settled what is Z, it would be probably easy to find out what is the counterpart

in medicine of the duration concept defined for the first time by Macaulay (in 1938) and independently by Redington (in 1952); see Formula (1).

Let us formulate the following hypothesis: the higher values (levels) of Z, the more healthy is a human body (a human body organ).

In the financial immunization context, there is a fixed date q when BP must attain at least a certain value L, called liability. In the medical context, one might say that there is a fixed date q when the quality of human health must attain at least a certain level L.

In the financial theory context, when interest rates s(t) change due to a shift a(t), that is, $s(t) \rightarrow s(t) + a(t)$, then the FV of BP at date q may fall below L dollars. In the medical context, the appearance of disease may cause a deterioration of health at date q.

We still do not know what should (could) be substituted for interest rates s(t), knowing that changes (movements, shifts) in interest rates mean a disease.

Using the concept of *duration* (and dedicated duration), we identified the set IMMU of all shifts (diseases) a(t) against which BP is immunized. By means of notion of duration and *convexity* (dedicated convexity), we determined the best immunizing portfolios for a large class of shifts (continuous functions). In the financial context, the best portfolios meant portfolios generating the highest (unanticipated) rate of return. In the medical context, the best would probably mean the fastest rate of health improvement.

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Immunization plays a key role in maintaining human health and each year, saves millions of lives from lethal pathogens and other fatal diseases in the most economical way, thanks to the advanced development of model vaccines. Subunit vaccines are regarded as a safer product than the whole microbe based-conventional vaccines and can be entrapped in various nanocarriers to form a vaccine adjuvant-delivery system (VADS) able to further boost their immunostimulatory activity. In this book, six groups of authors introduce immunization advances in VADSs designed for infection prophylaxis and cancer immunotherapy, problems and their resolution in both human and poultry immunization, and also, the mathematical model for assay of the basic immunization problem (BIP) understood from a finance point of view.

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